UNIVERSITY OF ALBERTA

Factors Affecting the Pharmacometrics of Etodolac

BY

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©

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled **FACTORS AFFECTING THE PHARMACOMETRICS OF ETODOLAC** submitted by **Brian W. Corrigan** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmacokinetics).

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Abstract

Etodolac (ET) is a COX II selective chiral NSAID, marketed as the racemate. Two factors that could affect the pharmacokinetics and toxicity of ET were studied in the Sprague-Dawley rat: The effect of sex and sex steroids, and the effect of altering enantiomeric ratio.

Following *iv* and *po* dosing to male and female rats, minimal sex differences were seen in the pharmacokinetics of ET. The largest differences were observed in the increased renal excretion of the conjugated form of the active S-ET in females. These differences were reversible, and appeared to be under the control of the hypothalmic-pituitary axis, primarily by the absence or presence of testosterone. Sex differences in intestinal permeability to ^{51}Cr-EDTA were not observed, due in part to the small degree of difference between sexes in the disposition of the drug, and the relative safety of ET at the dose used. The male rat remains a suitable animal model for pharmacometric studies of ET.

The effect of altering enantiomer ratio on the physicochemical properties, pharmacokinetics, and pharmacodynamics of ET was studied in male rats. The melting point binary phase diagram of ET was typical of a crystalline addition compound. Solubilities of ET compounds of various enantiomer ratioswere inversely correlated with melting point. Octanol/water partitioning of non-saturated solutions of ET were altered in a pH dependent manner. The kinetics of ET following oral dosing with a fixed dose of S and varying amounts of R yielded linear kinetics for R-ET and no alterations in S-ET. There was a trend to higher unbound levels of S-ET with

increasing amounts of R-ET in spiked plasma. Tissue distribution of S-ET was altered in some tissues with increasing doses of R-ET. Following oral dosing with S-ET, permeability to ^{51}CR-EDTA was similar to or higher than that following racemate, and minimal for R-ET. Utilizing a unique pharmacokinetic/ pharmacodynamic link model developed in our lab, meaningful relationships between effect compartment concentrations and permeability were found.

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LIST OF ABBREVIATIONS AND SYMBOLS

APA	Arylpropionic acid
AUC	Area under the plasma concentration-time curve
AUC_{0-t}	Area under the plasma concentration time curve from 0 h to the last measured concentration.
$AUC_{0-\infty}$	Area under the plasma concentration-time curve from 0 h to infinity
C	Temperature in degrees Celsius
Cl	Total body clearance
Cl_{0-t}	Clearance calculated by the dose given divided by the AUC_{0-t}
Cl/F	Body clearance following oral dosing
cm	Centimeters
C_{max}	Maximum plasma concentration following oral dosing
Conglomerate	A 50:50 ratio of enantiomers present as a physical mixture of two distinct crystals
COX	Cyclooxygenase
DSC	Differential scanning calorimetry
EHC	Enterohepatic circulation
ET	Etodolac
F	Absolute bioavailability
g	Gram(s)
G.I.T.	Gastrointestinal tract
GD	gastroduodenal

h	Hour(s)
HPLC	High performance liquid chromatography
IB	Ibuprofen
ip	intraperitoneal
iv	intravenous
K	Temperature in degrees Kelvin
Kg	Kilogram(s)
KT	Ketorolac
L	Litres
M	Molar
min	Minute(s)
mL	Milliliter(s)
mmol	Millimole(s)
N	Normal
NSAID	Non-steroidal anti-inflammatory drug
po	oral
R	Gas constant (1.9872 cal deg $^{-1}$ mole $^{-1}$)
Racemate	A 50:50 ratio of enantiomers, irrespective of phase
r^2	Correlation coefficient
s	Seconds
S.D.	Standard deviation
S.E.M.	Standard error of the mean

T_{max}	Time to maximal plasma concentration following an oral dose
TNF-α	Tissue necrosing factor alpha
$t_{1/2}$	Elimination half life
True racemate	The crystalline addition compound formed from a 50:50 ratio of enantiomers
V_d	Volume of distribution
Vd/F	Volume of distribution after oral dosing
vs	Versus
α	Level of significance
β	Terminal elimination rate constant (two compartment model)
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar(s)
$\Sigma X_{u\,0\text{-}t}$	Cumulative urinary excretion from time 0 to time t (in h)

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1. Introduction

1.1 Rationale for the study of enantiomeric ratios

A fundamental way of looking at the world is to view all objects in terms of their chirality or achirality. All objects, irrespective of size, either have or lack the geometric property that is responsible for the non-identity with its mirror image: a hand is chiral for example; a cube is not.

Most individuals with exposure to organic chemistry principles are familiar with chirality. Traditionally, when we think of enantiomers, the two mirror images, we think of molecules that exist in two enantiomorphic forms, both with the same physical and chemical properties in an achiral medium, such as melting point, freezing point depression, and ultraviolet absorbance.

However, what is often understated is the far reaching implication that chirality has in the biological world. Most animals upon visual inspection, exhibit a plane of symmetry. This sort of chirality is present to the molecular level in most plants and animals. Amino acids, proteins, and membranes all exhibit characterisitics of chirality.

As a result of the chiral nature of the biological matrix, The possibility for a number of stereoselective interactions with xenobiotics is possible. For many chiral molecules, the body has the capacity to act as a highly selective stereo-discriminator. The effect of a molecule on the body (pharmacology) may be stereospecific. The enantiomers of carvone for instance can elicit unique sensations, one enantiomer being

spearmint, the other essence of caraway. Glucose is another example, where only one of the 32 possible stereoisomers (α-D-glucose) is useful as an energy source for the body. Equally, the effect of the body on the molecule (pharmacokinetics) can be stereoselective, as has been determined for a number of chiral agents (Jamali *et al.*, 1989).

It has long been known that many xenobiotics are chiral. Historically, these chiral compounds were prepared from natural sources, and as a result were optically pure. However, due to the increased chemical synthesis of chiral drugs, many are now sold as the racemate, an equal proportion of both enantiomers.

In the last decade, the growing awareness that the two enantiomers of drugs may behave uniquely both quantitatively and qualitatively *in vivo* has intensified research efforts into both pharmacological and pharmacokinetic differences of enantiomers (Jamali *et al.* 1989). To date, almost all pharmacokinetic and pharmacological research has studied either each enantiomer administered alone or in a 50/50 ratio. These studies have yielded valuable information about bioinversion of xenobiotics, stereoselectivity in absorption, distribution, clearance and elimination, as well as stereoselectivity in pharmacological actions.

An often overlooked fact, however, is that the body is an environment where the enantiomer ratiosmay change with time. Most processes in the body are stereoselective, not stereospecific, and as a result, competition between enantiomers may occur at every level of the drug's disposition throughout the time course of the drug in the body. The two enantiomers may exhibit orders of difference in their

concentrations either in plasma or at sites of action in tissue, resulting from stereoselective clearance and/or tissue uptake. The resultant pharmacological effects at any time are thus derived from a combination of factors, depending on the ratio of enantiomers present, the relative potency of each enantiomer, and if applicable, the relative affinity of each enantiomer for the drug receptor at the site of action.

Almost invariably, interaction between enantiomers *in vivo* is studied by administration of each enantiomer alone compared to the racemate. Results from single enantiomer administration act as a baseline (assuming no inversion from one enantiomer to the other occurs), and are then compared to results from the racemate. Stated in other terms, almost all conclusions about possible effects of enantiomeric interaction on the pharmacokinetics and the pharmacology of chiral drugs *in vivo* have been determined by single point, fixed (equimolar) ratio studies.

The question that logically arises from this situation is; "What effect does altered enantiomer ratiosplay in the pharmacometrics of chiral compounds?" The effects of altered enantiomer ratioon physicochemical properties of compounds has been studied from a general chemistry perspective (Jacques *et al.*, 1981). However, from the pharmaceutical perspective, little or no attempt has been made to answer this question. To our knowledge, for only one drug, indacrinone (where the enantiomers each have unique pharmacological actions) have attempts other than purifying enantiomers been made to alter enantiomer ratio to improve therapy (Tobert *et al.* 1981). There is no understanding of how enantiomeric ratios other than racemic or single enantiomer may influence the pharmacodynamics or pharmacokinetics of a drug.

1.2 Rationale for using ET as a model

Etodolac (ET) (Lodine, Ultradol; Wyeth Ayerst, New York, NY) is an NSAID originally synthesized in the 1970s by Ayerst Laboratories in Montreal, Quebec. The drug proved to have the most favorable ratio of anti-inflammatory to deleterious effects from a series of compounds of 1-ethyl and 1-n-propyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-acetic acid derivatives synthesized. The initial pharmacological and pharmacokinetic work done proved ET to be both a safe and effective drug for various indications. Ironically, ET went on to be licensed in a number of different countries in western Europe and the United States, but only recently has been licensed for sale in Canada.

During the mid 1980s, a growing awareness of the chiral nature of many xenobiotics and the subsequent possibility for stereoselective or stereospecific processes in the chiral medium of the body lead to development of chiral assay techniques for various drugs, including ET. In 1986, a gas chromatography assay method for the stereospecific analysis of ET enantiomers was published utilizing pre-column derivatization techniques (Singh *et al.*, 1986). This was followed by a more convenient high performance liquid chromatograpy (HPLC) method in 1988 (Jamali *et al.*, 1988). Subsequently, many important factors were characterized in the stereoselective disposition of ET in humans, and a suitable animal model was identified for further studies into the mechanisms involved in the disposition of ET (Brocks and Jamali, 1994; Brocks and Jamali, 1990; Brocks, 1993). From this animal model work,

knowledge about the plasma pharmacokinetics, protein binding, tissue distribution and binding, and enantiomeric interaction are all available for ET (Brocks, 1993).

The underlying knowledge gained on ET coupled with its unique nature make ET an ideal candidate for study of the effect of enantiomer ratio on the pharmacometrics of NSAIDs. Unlike most other NSAIDs, plasma ratios of ET in humans and in the rat model show that the pharmacologically less active R-ET greatly exceeds S-ET. This predominance of the less active enantiomer allows for alteration of ratio while maintaining assay sensitivity. Preliminary work also suggested that ET forms a true racemate (Sattari and Jamali, unpublished data), similar to ibuprofen and other chiral arylpropionic acid derivatives in the NSAID family (Dwivedi *et al.* 1992). Equally important is the fact that ET has shown no enantiomeric bioinversion either *in vivo* or *in vitro*, a potential confounder for these studies (Brocks and Jamali 1990). Furthermore, a convenient and sensitive assay capable of being used for various biological tissues and fluids is available in our laboratory.

1.2.1 Rationale for sex studies

The classic animal model for sex differences in drug disposition is the rat. Usually males have a greater metabolic capacity than females, but in some steroid related compounds, the opposite is true (Skett, 1988). There are two postulated mechanisms for sex-related regulation of xenobiotic metabolic enzyme activity.

The first mechanism involves enzyme "imprinting" during the perinatal period before birth. The brain is imprinted to show a sexual dimorphism in its ability to produce certain xenobiotic metabolizing enzymes. This enzyme imprinting is largely due to the organizational effects of gonadal steroidal hormones (Bonate, 1991; Maiter *et al.*. 1991). Early imprinting of several neuroendocrine functions by the perinatal presence or absence of gonadal hormones has been demonstrated in the rat. (Maiter *et al.*. 1991) This includes size and cell population of the hypothalamic nuclei, and activity of specific neurotransmitter and neuropeptide networks, as well as sensitivity of hypothalamic neurons to hormonal stimulus (Bonate, 1991). While testosterone appears to be the main determining factor in this regulation, ovaries can antagonize some androgen effects (Bonate, 1991).

The second mechanism is through androgenic maintenance of certain enzymatic activities via the hypothalamic-pituitary- gonadal axis. A great deal of the differences seen between male and female patterns of metabolism can be accounted for by quantitative and qualitative differences in P450. To date, most of our information comes from rat studies. In general, males rats have a 10-30% greater amount of hepatocellular P450 than females (Kobliakov *et al.*. 1991). This leads to a larger amount of almost all types of hydroxylation reactions in adult male rats until 104 weeks, after which there are no discernable differences between male and female liver oxidative function.

For ketoprofen, also a chiral NSAID, it has been shown that the female rat serves as a more suitable animal model than the male rat (Palylyk, 1994). This was

mainly due to the fact that in the females, the main route of elimination of ketoprofen is via the acyl-glucuronide which subsequently is excreted in urine. This pattern is similar to that observed in humans. Preliminary observations by Brocks (1993) also suggest the possibility of sex differences in the disposition of ET in the rat (Brocks, 1993). It was observed that females excreted the conjugated metabolites of ET into urine, similar to humans. It was therefore important, to further investigate the influence of sex on the pharmacokinetics of etodolac in search for a more suitable animal model. The stereospecific pharmacokinetics of ET in female and male rats and a determination of whether the mechanism underlying the sex differences for this drug were "imprinted" or under the control of the hypothalmic-pituitary axis was examined. As well, the effect of sex and sex hormones on ^{51}Cr-EDTA permeability following oral dosing was also assessed to determine which sex was suitable as an animal model for G.I.T. toxicity.

1.2.2 Review of ET

Physical properties

1.2.1.1 Absolute configuration and crystalline form (racemate)

The more active enantiomer of ET was assigned the S configuration following crystallographic analysis of the (S)-(-)-borneol ester of (-)- etodolac (Humber *et al.* 1986). The conformation of (-)- ET was determined by crystallographic analysis of racemic ET. It is now known that racemic ET forms anhydrous orthorhombic crystals

belonging to the space group Pbca with unit cell dimensions of a = 8.602, b = 18.586, c = 19.055 Angstroms (Humber *et al.* 1986).

1.2.1.2 Stability

The stability of ET has been shown to be dependent upon temperature and pH. First order degradation of ET is observed with high temperature (> 85 °C) or low pH (only with high temperature) (Lee *et al.* 1988). All degradation products involve decarboxylation of the acetic acid moiety. Three kinetically equivalent processes have been postulated for this phenomena.

1. Unimolecular C_1-O bond fission prior to decarboxylation
2. Six member ring formation by intramolecular hydrogen bonding between the carboxyl OH and the pyrano ring oxygen causes a simultaneous decarboxylation and ring opening process
3. A bimolecular attack of water on the carboxyl OH.

Evidence of degradation of stock solutions of ET was determined by HPLC analysis. At refrigerator or room temperature and under the slightly alkaline pH conditions and time periods that were used for ET solutions throughout this study, evidence of ET degradation was not present.

Pharmacological properties

1.2.2.1 Mechanism of action

The pharmacological and toxicological properties of ET have been extensively studied, and many of the underlying mechanisms responsible for these actions have been identified (Table 1). Similar to other NSAIDs, one of the primary mechanisms believed to be involved in the activity of ET is inhibition of cyclooxygenase (COX). It was found that the (+)- enantiomer possessed most of the anti-inflammatory activity, as measured by prostaglandin synthetase inhibition and reduction in paw swelling in rats with adjuvant polyarthritis (Demerson *et al.*, 1983). Recently different isoforms of COX have been identified. COX-1 is constitutive, and present under normal physiological conditions. COX-2 is cytokine inducible, typically by inflammatory processes *in-vivo* (Seibert *et al.*, 1995). ET appears more selective for the non-constitutive COX-2 enzyme sub-type associated with G.I.T. toxicity (O'Neill *et al.*, 1995).

1.2.2.2 Clinical efficacy

Years of use in various countries have proven that ET shows clinical efficacy for a variety of conditions in humans (Table 2). Etodolac has proven to be a well tolerated and safe drug compared to various other NSAIDs (Table 3), yet similar to other NSAIDs, G.I.T. complaints remain the dose limiting toxicity. These may relate, in part, to inhibition of COX, the enzyme responsible for the generation of mucosal protective prostanoids (Melerange *et al.*., 1994).

Table 1 Potential Mechanisms for Pharmacological and Toxicological Manifestations of ET

Mechanism	Reference
Inhibition of COX	Demerson *et al.* 1983
Suppression of type II collagen induced arthritis (mice)	Inoue *et al.* 1993
Inhibition of prostaglandin E_2 (animal and *in vitro*)	Henrotin *et al.* 1992 Inoue *et al.* 1991a, Melarange *al.* 1994,Lee and Dvornik, 1985
Inhibition of granuloma formation (animal)	Inoue *et al.* 1991a
Inhibition of leukocyte function (rat)	Inoue *et al.* 1991a Gervais *et al.* 1984
Inhibition of bradykinin release (rat)	Inoue *et al.* 1991b
Decreased synthesis of glycosaminoglycan (rabbit and *in vitro*)	Redini *et al.* 1990
Maintenance of type II collagen expression by chondrocytes (*in vitro*)	Goldring *et al.* 1990
Inhibition of serum TNF -α levels (mice)	Ochalski *et al.* 1993
Not lipoxygenase inhibition linked (*in vitro*)	Sirois *et al.* 1984

Table 2 Studies of ET efficacy in Humans

Efficacy Shown for	Reference
Osteoarthritis	Grisanti *et al.* 1992; Astorga Paulsen *et al.* 1991; Karbowski, 1991; Puccetti and Ciompi, 1995; Porzio, 1993a; Williams *et al.* 1989; Ciocci, 1989
Rheumatoid Arthritis	De Queiros, 1991; Dick *et al.* 1993; Schattenkirchner, 1991; Lonauer *et al.* 1993; Porzio, 1993b; Ciompi *et al.* 1995
Sports Injuries	D'Hooghe, 1992
Degenerative Joint Disease	Dick *et al.* 1992; Khan and Williams, 1992; Todesco *et al.* 1995
Gout	Maccagno *et al.* 1991
Acute Lumbago	Arriagada and Arinoviche, 1992
Post-operative Pain	Pena, 1990
Dental Pain	Gaston *et al.* 1984; Giglio and Campbell, 1986; Scott *et al.* 1986; Gaston *et al.* 1986

Table 3 ET Toxicity Studies in Humans

Toxicity Observed	Results	Reference
Gastrointestinal toxicity	Less than naproxen	Leese, 1992; Schattenkirchner, 1993, Bianchi Porro *et al.* 1991; Taha *et al.* 1989; Russell, 1990
	Less than ASA, indomethacin, ibuprofen, and piroxicam	Lanza and Arnold, 1989; Lanza *et al.* 1987; Karbowski, 1991
	Less than or equal to piroxicam	Dick *et al.* 1993
	Equal for sustained and regular release	Dreiser, 1993
	Excellent global assessment	Karbowski, 1989
Agranulocytosis	Case report	Cramer *et al.* 1994
Acute Hepatitis	Case report	Latrive *et al.* 1992
Vasculitis	Case report	Willemin *et al.* 1989

Pharmacokinetic properties

The stereospecific pharmacokinetic properties of ET have been thoroughly studied and show many similarities between humans and rats, with qualitative similarities in metabolic patterns observed (Table 4). Various previously published reviews of ET pharmacokinetics in humans and rats are available (Brocks and Jamali, 1994, Brocks and Jamali, 1991; Brocks and Jamali, 1990).

Briefly, in humans, ET is similar to many other NSAIDs in that it is well absorbed (F 0.7-1.05), highly protein bound, and extensively metabolized. Glucuronide conjugates are present in urine and plasma, with bile being a minor route of elimination. Unlike most other chiral NSAIDs, plasma concentrations of the less active R are predominant. Stereoselectivity is observed, with R > S for C_{max}, and AUC, and S > R for CL/F , Vd_{β}/F, and unbound fraction. Age *per se* is not a factor in the pharmacokinetics of ET, but renal function is, due to the large degree of conjugated metabolites of ET eliminated in urine. Considerable amounts of both ET and ET conjugates are present in synovial fluid, the proposed site of action of ET (Brocks and Jamali, 1994). Interaction between enantiomers was not observed after *iv* dosing of 5 mg/Kg of racemic ET, and 2.5 mg/Kg of each enantiomer (Brocks and Jamali, 1991; Brocks and Jamali, 1990).

The disposition of ET in rats is similar to that in humans (Table 4), with a few notable exceptions. Marked enterohepatic circulation (EHC) is observed in the rat, due to metabolism to glucuronide conjugates and clearance into bile, with S conjugate in

bile more predominant than R (Brocks and Jamali, 1990). As well, urine is a minor route of elimination in the rat as compared to humans, especially in male rats (Brocks, 1993). Both species share similar oxidative and conjugated metabolites.

1.3 Hypotheses

1) Altering enantiomer ratio of ET, may alter;

a) physicochemical properties of ET,

b) pharmacokinetic properties of ET,

c) pharmacodynamic properties of ET.

In light of preliminary findings (Brocks, 1993) of gender dimorphism of ET in excretion of conjugates into urine, it was also deemed necessary to first determine which gender is best for the study of the pharmacometrics of ET. Thus a secondary hypotheses was

2) Sex differences in the disposition of ET may exist in the Sprague Dawley rat and are a function of the hypothalmic-pituitary axis, primarily sex steroids.

1.4 Objectives

1.4.1 Sex studies

1.4.1.1 Determine the degree of sex differences in the pharmacokinetics of ET.

The effect of gender on the pharmacokinetics of ET was studied following *iv* and oral dosing in rats to determine the extent of sex differences.

1.4.1.2 Determine the effect of sex hormones on the pharmacokinetics of ET

Androgens play a significant role in the mechanism of sex related differences in metabolism.They are postulated to work at two different levels 1) via imprinting of sexual identification in the neonatal brain, which eventually affects the ability to respond to androgens and the enzyme activity in the adult and 2) to maintain certain enzyme activities in the mature animal via the hypothalmic-pituitary-gonadal axis (Bonate, 1991)

The effect of sex hormones on the differences seen in the disposition of ET was studied to determine if sex differences were a result of sexual imprintation or the hypothalmic-pituitary-gonadal axis.

Table 4 Summary of Stereoselective Disposition and Metabolism of ET in Humans and Rats.

Parameter	Humans	Rats
AUC	S <<< R[2]	S << R[2]
Volume of distribution	S >>> R[2]	S >> R[2]
Cl	S >>> R[2]	S >> R[2]
Unbound fraction	S > R[3]	S > R[3]
Elimination rate (K_E)	S ≥ R[2]	S > R[1]
Renal clearance	no differences (9-22%)[2]	none in males[1]
Absorption	rapid [2]	not determined
Bioavailability	0.8 - 1.0[5]	not determined
Primary route of elimination	urine[2]	feces via bile[1]
Metabolism	not determined	not determined
S-ET	primarily oxidized[6]	Primarily conjugated[1]
R-ET	primarily oxidized[6]	oxidized and conjugated[1]
Effect of age	not significant[2]	not determined
Effect of cholecystectomy	delayed absorption[2]	not determined
Conjugates in plasma	Yes[2]	No (males)[1]
Induction with phenobarbital	not determined	Increased oxidation[6]
Inhibition with cimetidine	not determined	no effect[4]
AUC due to EHC	not determined	75 % and 26 % (S and R)[1]
Evidence of inversion	not determined	No[1]
Synovial/Plasma ratio	> 1 (for S-ET)[3]	not determined
Conjugates in Plasma	Yes[2]	No[1]

1.Brocks and Jamali, 1990
2.Brocks *et al.*, 1992
3.Brocks and Jamali, 1991
4. Brocks, 1993
5. Cayen *et al.*, 1981
6. Brocks and Jamali, 1992
7. Ferdinandi *et al.*, 1986

1.4.1.3 Determine if there is sex differences in the toxicity of ET.

The incidence of G.I.T. side effects with NSAIDs has been reported to be as high as 11%, with females having a higher incidence of G.I.T. side effects from NSAIDs than men. (Famaey and Paulus, 1992). It has been suggested that the disposition of NSAIDs in the body plays an important role in their overall toxicity, raising the question of whether sex dimorphism in NSAID disposition may be responsible for these differences in toxicity. A previously established method utilizing permeability to ^{51}Cr-EDTA as a surrogate measurement of intestinal toxicity was used to determine the effect of sex and sex hormones on the intestinal toxicity of ET.

1.4.2 Ratio studies

1.4.2.1 Physicochemical

Determine changes in melting point of ET

The melting point is a physical property that may be important in determining the disposition of a compound. Melting points of ratios of ET compounds formed were measured (in 5 % intervals) to determine if the enantiomer ratioaffects the physical characterisitics in the solid phase.

Determine thermal characteristics of ET compounds

DSC was performed on various compounds of ET to further characterize changes occurring in the crystalline state following alteration of enantiomer ratio.

Determine changes in solubility resulting from various ET ratioss

The solubilities of compounds at inflection points in the binary phase diagram of ET were measured to determine the effect that alteration of enantiomer ratio has on the properties of enantiomers in solution.

Determine changes in partition coefficient resulting from various ET ratios

The partition coefficients of ET compounds were measured at various pH to determine if enantiomer ratioaffects partition coefficient and if so, whether it occurs at physiologically relevant pHs.

Define the characterisistics of ET compounds' ultraviolet spectra

The absorbance and γ_{max} in aqueous solution over the pH range of 2 to 11 were measured in triplicate for enantiomer ratios of ET to determine if interactions in solution were occuring.

1.4.2.2 Pharmacokinetic studies

Determine the effect of altering enantiomer ratio on the pharmacokinetics of ET

The plasma pharmacokinetics of a fixed dose of S-ET with varying amounts of R-ET after an oral dose was studied in male rats.

Determine if tissue distribution is altered for ET compounds by varying enantiomeric ratio

The effect of altering enatiomeric ratio on tissue distribution was examined at a fixed time point for a fixed dose of S and varying amounts of R-ET in male rats.

Determine if plasma protein binding of S-ET is altered by the presence or absence of R-ET.

The effect of altering enantiomer ratioon the protein binding for a fixed concentration of S-ET and varying amounts of R-ET was studied in plasma.

1.4.2.3 Gastrointestinal toxicity

Determine ^{51}Cr-EDTA time course of permeability for S, R, and racemate

The time course of ^{51}Cr-EDTA permeability following oral dosing with S, R, and racemic ET was measured to determine if enantiomeric interaction occurs in the toxicity of ET and to determine a suitable time for further single time point studies of various ratios.

Determine if there is a relationship between concentrations of ET and ^{51}Cr-EDTA Permeability changes

The relationships between plasma concentrations and effect and effect compartment concentration and effect (utilizing a pharmacokinetic/pharmacodynamic link model) were studied to determine if meaningful relationships between concentrations of ET and ^{51}Cr-EDTA permeability could be determined.

Determine the effect of altering enantiomer ratioon ^{51}Cr-EDTA permeability

The effect of altering enantiomer ratioon ^{51}Cr-EDTA permeabilty was examined using a fixed dose of S-ET and varying amounts of R-ET at a single time.

2. Sex

2.1 Background

There has been a growing awareness that both qualitative and quantitative sex differences exist for the disposition of many drugs, including NSAIDs (Skett, 1988, Wilson, 1984, Bonate, 1991). A well known example from the NSAID class that displays sex dimorphism in man is ASA. Acetyl-transferase, which metabolizes acetylsalicylic acid, has been found less active in women than in men (Famaey and Paulus, 1992). In addition, men display a slower oral but a more rapid intramuscular absorption rate for ASA (Trnavska and Trnavsky, 1983; Aarons *et al.* 1989). Further, men consequently display lower AUC and an increased elimination rate after oral doses of ASA (Trnavska and Trnavsky, 1983).

Sex differences in the disposition of NSAIDs in the rat model have also been observed. For example, ketoprofen, shows marked sex differences in the pharmacokinetics in Sprague Dawley rats. Significantly higher plasma concentrations of both enantiomers and substantially greater urinary excretion of the glucuronide conjugate of S-KT has been observed in females, making it a more suitable animal model than the male rat (Palylyk and Jamali, 1992).

Limited information is available regarding the effect of sex on the disposition or toxicity of ET enantiomers (Brocks and Jamali, 1994). Most of the pharmacokinetic studies of ET completed in animals have not included females, or in humans, had failed to compare sex in the subjects used in the study. A preliminary study by Brocks in

rats(1993) showed some sex related differences in the kinetics of ET, specifically, in the renal excretion of conjugated ET into urine. The purpose of these studies in Sprague Dawley rats was to examine sex differences in the disposition of ET in the rat and to determine which sex was most appropriate for further study of ET. The permeability to ^{51}Cr-EDTA after oral dosing with ET, a surrogate measure of toxicity, was also studied to determine if both sexes exhibited similar permeability characteristics.

2.2 Materials and Methods

2.2.1 Chemicals

Racemic ET, as well as R and S-ET enantiomers were provided by Wyeth-Ayerst Laboratories (New York, NY, USA). Internal standard [(±) -2-(4-benzoylphenyl)butyric acid], was provided by Rhone-Poulenc (Montreal, PQ, Canada). Isopropyl alcohol, ethyl chloroformate, isooctane, HPLC grade chloroform, HPLC grade water, H_2SO_4, NaOH, and diethyl ether were purchased from BDH chemicals. Methoxyflurane (Metofane) was obtained from Pitman-Moore Ltd (Mississauga, Canada), polyethylene glycol 400 from Fisher Scientific (Edmonton, Canada), heparin (Hepalean) from Organon Teknika (Toronto, Canada). Estradiol valerate (Delestrogen) was purchased from Squibb (Montreal, Canada) and testosterone cypionate (Depotestosterone) was purchased from Upjohn (Don Mills,

Ontario, Canada). ^{51}Cr-EDTA was purchased from Dupont,Wilmington, Delaware (specific activity 570 MCi/mg).

2.2.2 Analysis of etodolac enantiomers in biological fluids

Plasma and urine extraction

Concentrations of etodolac in plasma and urine were determined based on a normal phase HPLC method (Jamali *et al.* 1988). The method consisted of addition of IS ((±)-2-(4-benzoylphenyl)butyric acid) to samples followed by extraction into a mixture of iso-octane:isopropanol (95:5). The organic layer was evaporated to dryness.

Alkali labile conjugates of ET in urine were cleaved by addition of 1 M NaOH and subsequent acidification prior to extraction. ET conjugates were calculated by the difference in ET concentrations between hydrolysed and unhydrolysed samples.

Derivatization of extracted ET and IS

Following extraction from biological fluids, derivatization to form diastereoisomers of IS and ET was performed by sequential use of ethyl chloroformate and R-(+)-α-phenylethylamine (BDH Chemicals)to allow formation of the diastereoisomer. R-(+)-α-phenylethylamine was used rather than S to allow S-ET, the active enantiomer, to elute earlier, and thus improve sensitivity of the assay. Furthermore, S-ET is present in much lower concentrations in plasma after a racemic

dose, causing further need for an increased assay sensitivity for S-ET (Brocks, 1993). Derivatized ET and IS were extracted into chloroform and evaporated to dryness.

Chromatographic conditions

The diastereoisomers formed by the derivatization process were dissolved in 0.2 ml of mobile phase consisting of hexane:ethyl acetate:isopropanol (85:15:0.2), and injected into an HPLC system consisiting of a Waters 712 Wisp, Waters LC pump, and Lambda-max model 481 LC-spectrophotometer (Waters, Canada).

2.2.3 Surgical Procedures

Jugular vein cannulation

Catheterization of female and male rats at the right jugular vein was performed using silastic tubing (0.58 mm i.d. x 0.965 mm o.d.; Clay Adams, Parsippany, NJ). Animals were allowed to recover overnight and had access to water *ad libitum*. They were fasted overnight until 3 h post dose.

Gonadectomy

Castration was performed by making a small median incision of about 1 centimeter through the skin at the top of the scrotum. A small incision was made into the scrotal sac and a single ligature was made round the blood vessels and the vas deferens, followed by a distal cut allowing removal of the testis and epididymis. Incisions were closed using tissue glue (Nexaband liquid, Tripont Medical, Raleigh, NC). Bilateral ovariectomy in female rats was performed via small midline dorsal

incisions with ovaries severed at the junction of the fallopian tube and uterine horn following ligation. The muscular layer was sutured and the skin clipped with 9 mm wound clips (Autoclips, Clay Adams, Parisippanny, NJ), which were removed after three days. All surgery was performed under methoxyflurane induced anesthesia (Pitman-Moore Ltd., Mississauga, Canada). All animals were housed individually in plastic metabolic cages and allowed freedom of movement for the duration of the experiments. They were subjected to a 12 h light/dark cycle throughout the study.

2.2.4 Dosing and sample collection

All ET doses in the pharmacokinetic studies throughout were dissolved in polyethylene glycol 400 (PEG400) and administered at 9 am on the day following surgery. Althought there is some evidence that PEG 400 may alter the disposition of some drugs, earlier authors not using PEG as a diluent reported similar pharmacokinetic estimates in the rat model(Inoue *et al.*, 1991). Intravenous doses were given into the jugular vein cannula followed by 0.2 ml of 100 unit per ml heparinized saline. Oral doses were administered using a 18 gauge gavage needle. Blood (0.2ml) was collected from the jugular vein cannula following ET administration. The catheter was flushed with 0.2 ml of 100 units per ml heparin following dosing and after each blood sample collection. Immediately following collection, plasma was separated from blood by centrifugation at 1800 g for 3 min using a microcentrifuge (Fisher model 235A, Fisher Scientific, Edmonton, Canada). Total urine output was collected in acid rinsed containers and pooled for 36 h. All samples were stored at -20°C until analyzed.

2.2.5 Lower intestinal toxicity

Procedures and apparatus

Male Sprague-Dawley rats were housed at ambient temperature and humidity in individual metabolic cages with wire mesh floors, allowing for separate quantitative collection of urine and feces. Animals were fed a standard rat chow the day before and 3 h following dosing, and allowed free access to water for the duration of the experiment.

To test intestinal permeability, 0.5 ml of a solution containing 10 μCi/ml of ^{51}Cr complexed with EDTA was administered orally following the dose of placebo or ET. Each ET compound was dissolved in PEG 400 and was administered orally through an 18 gauge 5 cm curved feeding needle (Harvard Apparatus) attached to a 1 ml syringe at 9 a.m. Urine was collected 0 to 8 h following the administration of the ^{51}Cr-EDTA solution. The urine was collected in cups containing 1 ml of 1 M H_2SO_4 to inhibit microbial growth. After each collection period 10 ml of tap water was used to rinse the urine collection trays and the total contents of the cup was then transferred to scintillation vials. Each experiment contained placebo (PEG) treated animals as baseline controls to minimize inaccuracy from fecal radioactive contamination.

Urine samples were counted by a gamma counter Beckman Gamma 8000 (Irvine, California) for 1 minute in a counting window scanning within a range of 0-2 Mev. At least two standards of 100 μl of the administered ^{51}Cr-EDTA solution were counted with every set of urine samples. Relative permeability was determined by calculating the activity present in each urine sample as a percent of the administered dose after correcting for background radiation.

2.2.6 Sex studies design

Pharmacokinetics

Rats were divided into 4 groups (n=4 for each group). Orally dosed females, orally dosed males, i.v. dosed females and i.v. dosed males. Males and females in each category (oral and i.v.) were paired with respect to time and date of dosage administration. Racemic ET (5mg/kg) was administered to each of the groups. Intravenous doses were given into the jugular vein cannula.

Gonadectomy disposition studies

Rats were divided into 6 groups, each consisting of 4 animals per group; females, males, ovarectomized females (OV), castrated males (CA), ovarectomized plus testosterone (OV+T), and castrated plus estrogen (CA+E). Rats were dosed with either estradiol valerate (1mg/kg) (CaE) or testosterone cypionate (10 mg/kg) (OvT) via the i.m. route weekly for 8 weeks. The treatment time was sufficient to allow washout of both the presence and the effects of pre-exisiting sex steroids). Eight weeks post-surgery, the rats were dosed with 5 mg/kg of racemic ET dissolved in polyethylene glycol 400. Urine was collected from 0 to 24 h after ET administration. All specimens were frozen at -20° C until analyzed.

Toxicity studies

Rats (6 groups as in the previous study, 4 rats per group) were placed in individual metabolic cages with wire mesh floors allowing for selective quantitative collection of urine and feces. Animals were fasted overnight then were allowed free access to food (standard rat chow) three h post-^{51}Cr-EDTA dosing. Water was

supplied for the duration of the experiment. To test baseline intestinal permeability, a solution containing ^{51}Cr-EDTA in water was administered orally to each individual rat through an 18 gauge 5 cm curved feeding needle attached to a 1 ml syringe. Urine was collected in acid rinsed cups 0 to 8 h following the administration of the ^{51}Cr-EDTA solution to act as a baseline. Gamma emission radioactivity (0-2 Mev), corrected for background and decay, were determined in a Beckman Gamma Counter and activity as a percent of the admininistered dose was calculated. The following day, the rats were dosed with 5 mg/kg of racemic ET dissolved in polyethylene glycol 400. Three h post-dosing, the animals were again dosed with ^{51}Cr-EDTA and urine was collected for 8 h. The percent of dose above baseline present in urine was reported.

2.3 Results and Discussion

2.3.1 Pharmacokinetics

The dispostion of ET in intact male and female Sprague Dawley rats demonstrated a limited degree of sex dimorphism, similar to that seen in humans. Table 5 depicts pharmacokinetic indices following i.v. and oral administration of 5 mg/kg of the racemate. In all groups, plasma concentrations of both enantiomers decreased for the first 3 to 4 h, followed by the appearance of a secondary peak (more pronounced for S-ET) between 3 and 6 h post-dosing. These fluctuations have been attributed to the large degree of enterohepatic recirculation that occurs, especially for S-ET, making half life an unreliable and variable parameter for ET. Similar fluctuations may have led to discrepancies in half life values reported earlier by other authors for ET (Brocks *et al.* 1990, Honda *et al.* 1991). As a result, we chose to report AUC_{0-36} rather than $AUC_{0-\infty}$.

Significantly larger AUC_{0-36} parameter estimates were seen for R-ET compared to S-ET in both sexes and after both administration methods. AUC_{0-36} for both enantiomers was significantly higher in females than males, except for AUC_{0-36} of R after i.v. dosing ($p=0.073$).

Oral plasma concentration-time profiles indicated rapid absorption in both females and males of both ET enantiomers (Figure 1) (T_{max} in females; 1.1 ± 0.4 h and 1.4 ± 0.5 h Males; 0.85 ± 0.7 h and 1.15 ± 0.8 h for S and R-ET respectively). Both males and females showed higher R-ET C_{max} values than S-ET (C_{max} female S 3.39 ± 1.3, R 13.54 ± 2.5; male; S 3.01 ± 1.7. R 13.79 ± 8.1 μg/mL). However, no significant sex differences were observed in C_{max} or T_{max} for either enantiomer.

The most marked sex differences seen were in the mean cumulative (36 h) urinary excretion of conjugated ET in rats (Table 5). Conjugated R in urine was below detectable limits in males. Cumulative amounts of conjugated S-ET in male urine were minimal. In contrast, females displayed at least 10 fold higher cumulative conjugated S-ET and R-ET in urine than that of males. For both routes of administration in females, S-ET was significantly higher than R. These results are consistent with earlier preliminary findings (Brocks, 1993).

The exact mechanism underlying this sex difference in the renal excretion of acyl-glucuronides of ET in urine remains unclear. There appears to be no direct glucuronidation of ET by the kidneys in males (Brocks and Jamali, 1991), even though evidence of glucuronidation for other NSAIDs of the 2-APA class such as ibuprofen exists for male rats (Ahn *et al.*, 1991). Glucuronidation of ET in female rat kidneys has not been determined. However, other NSAIDs, such as ketoprofen, have been observed to have higher glucuronide activity in the female kidney *in vitro* than male (Palylyk, 1994).

Table 5 Pharmacokinetic Parameters in Male and Female Rats after dosing with 5 mg kg of Racemic ET.

	AUC $_{0\text{-}36}$ S (μg*hr/ml)	**AUC $_{0\text{-}36}$ R** (μg*hr/ml)	**$\Sigma X_{u\ conjugate\ 0\text{-}36}$ S-ET** (% of enantiomeric dose)	**$\Sigma X_{u\ conjugate\ 0\text{-}36}$ R-ET** (% of enantiomeric dose)
I.V.				
Females	110.03±25.7[a]	381.70±112.1[a]	16.03±5.0[ab]	1.90±1.01[a]
Males	91.69±10.8[a]	326.06±84.22[a]	0.08±0.1[b]	b.d.
Oral				
Females	79.27±2.2[a]	253.42±54.1[a]	6.59±3.5[ab]	0.14±0.27[a]
Males	59.14±41.7[a]	199.75±112.2[a]	0.17±0.1[b]	b.d.

b.d. = Below detectable limit of the assay

[a] Significantly different than its antipode.

[b] Significantly different than the opposite sex.

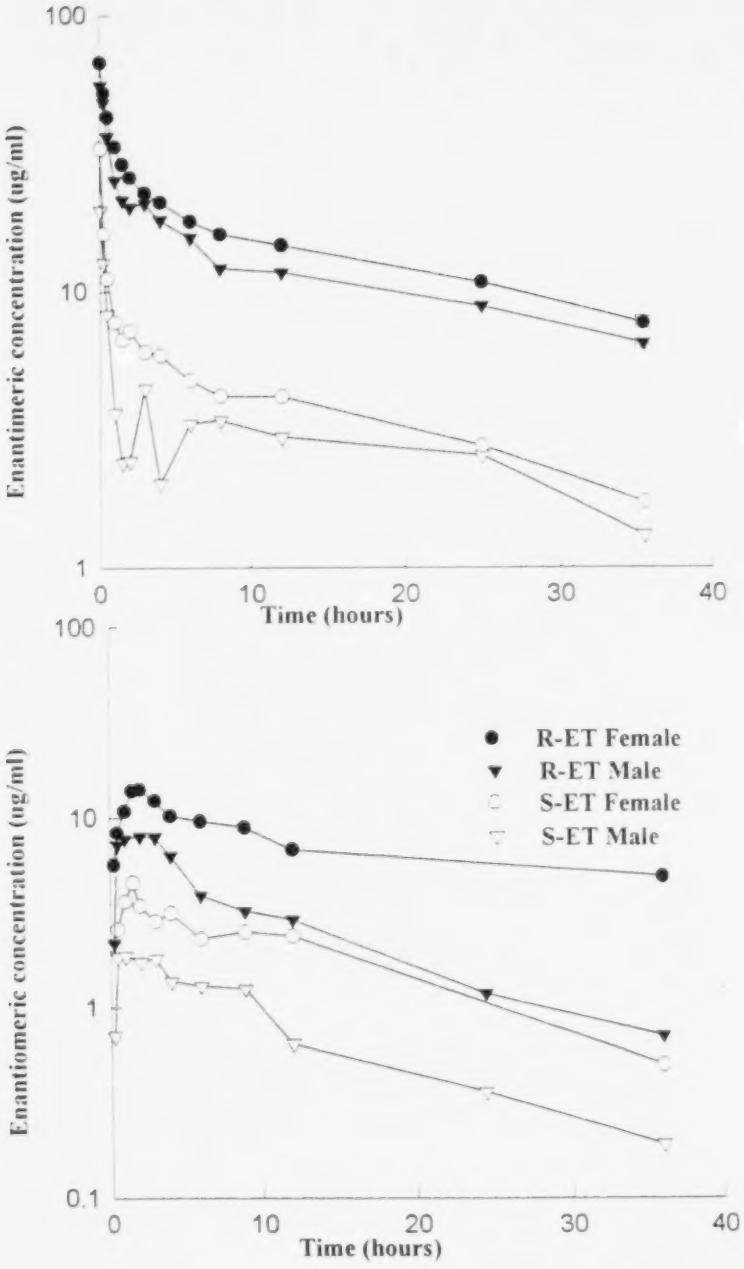

Figure 1. Representative plasma concentration vs time curve of ET after iv (top) and oral (bottom) dosing to male and female Sprague Dawley rats with racemic ET (5mg/kg).

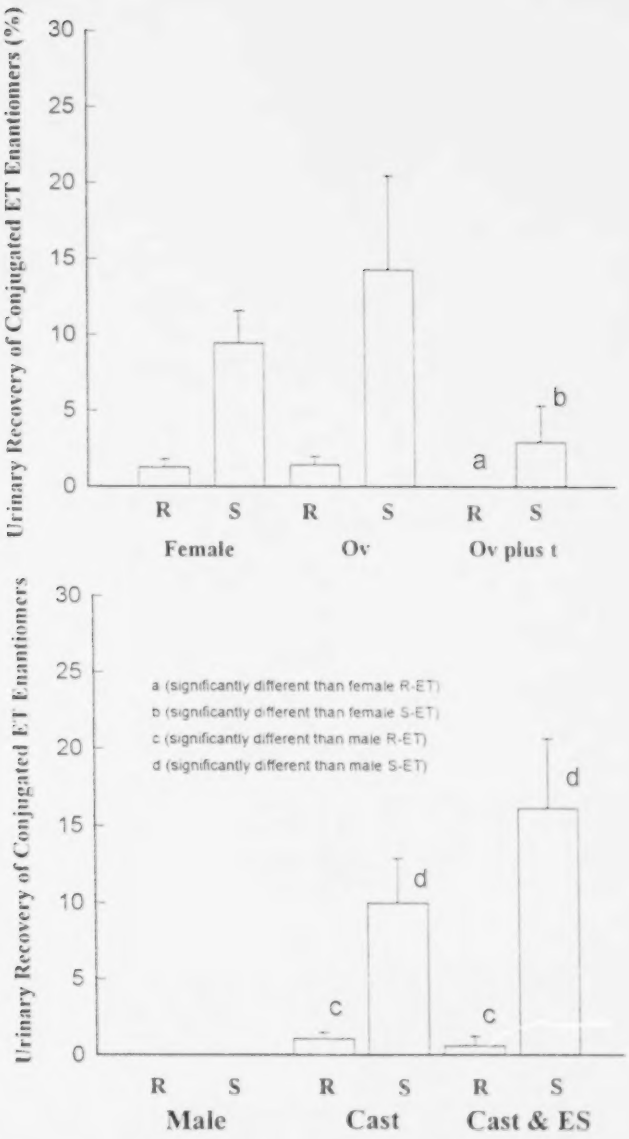

Figure 2. Cumulative 24 h urinary recovery of conjugated ET after 5 mg/kg oral dosing following gonadectomy and androgen supplementation. (% enantiomeric dose). Female, (normal adult females), OV, (bilaterally ovarectomized females), Ov plus T, (ovarectomized females plus testosterone), Male, (normal adult males), Cast, (castrated males), Cast – ES, (castrated males plus estrogen (n=4 for each group).

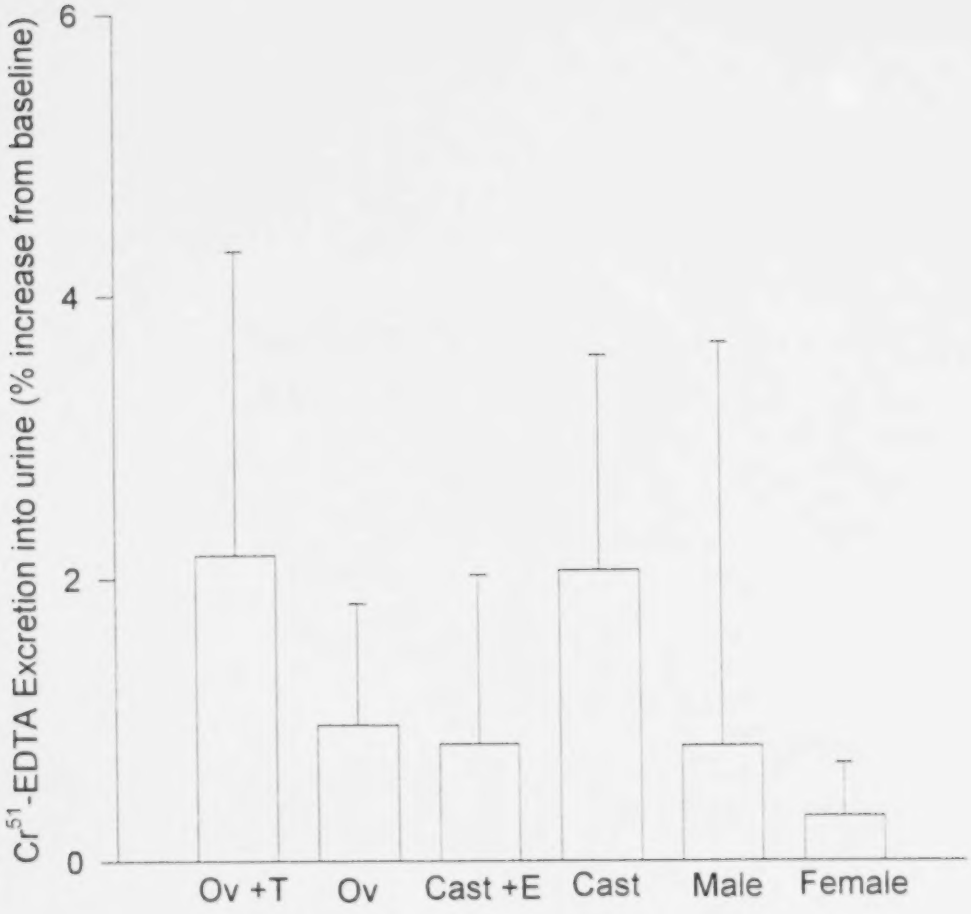

Figure 3. Percent of ^{51}Cr-EDTA dose excreted into urine following gonadectomy and androgen supplementation (above baseline). Female, (normal adult females), OV, (bilaterally ovarectomized females), Ov plus T, (ovarectomized females plus testosterone), Male, (normal adult males), Cast, (castrated males), Cast + ES, (castrated males plus estrogen)(n = 4 for each group).

The stereoselectivity observed in the urinary excretion of ET may be explained by a number of mechanisms. S-ET has a larger protein unbound fraction, resulting in greater amounts being available for clearance through the kidney (Brocks 1993). A second plausible mechanism is that female renal conjugation of ET is stereoselective in favor of S-ET, as is rat hepatic conjugation of ET in males (Brocks 1993). A third plausible mechanism may be that the diasteromeric nature of S and R ET glucuronides impose unique physical chemical characteristics resulting in differences in excretion into urine (Kroemer and Klotz, 1992). Iwakawa *et al.* reported data for carprofen glucuronides where the S-carprofen glucuronide urinary clearance exceeded that of R (Iwakawa *et al.*, 1989). Coadministration of probenicid led to an increase in the S/R ratio of the renal clearance, indicating that clearance of the S-carprofen glucuronide was more affected by probenicid co-administration than the R.

In addition, the possibility of sex dependent transport processes in either transport into or out of the kidney or in deconjugation of the glucuronide cannot be ruled out based on the available data.

2.3.2 Effect of androgens and gonadectomy on renal conjugates of ET

Following gonadectomy and administration of sex hormones, significant differences between treatments could be observed for the conjugated R-ET in urine. More interestingly, S-ET also showed significant differences between groups (Figure 2). Ovarectomy alone appeared to have little effect on the urinary excretion of conjugated S-ET, whereas the ovarectomy plus testosterone group excreted significantly less conjugated S-ET than female, a pattern similar to the male rat. Both castrated and castrated plus estrogen groups showed significantly higher levels of conjugated S-ET than males.

The two mechanisms postulated to be responsible for sex related differences in xenobiotic metabolic enzyme activity are 1) enzyme "imprinting" during the perinatal period before birth. and 2) androgenic maintenance of certain enzymatic activities via the hypothalamic -pituitary -gonadal axis. The first involves a sexual dimorphism in ability to produce certain xenobiotic metabolizing enzymes, largely due to the effects of gonadal steroidal hormones present before birth (Bonate, 1991; Maiter *et al.*. 1991). Early imprinting of several neuroendocrine functions by the perinatal presence or absence of gonadal hormones has been demonstrated in the rat (Maiter *et al.*. 1991). This includes size and cell population of the hypothalamic nuclei, and activity of specific neurotransmitter and neuropeptide networks, as well as sensitivity of hypothalamic neurons to hormonal stimulus (Bonate, 1991). While testosterone appears to be the main determining factor in this regulation, ovaries can antagonize some androgen effects (Bonate, 1991). The second mechanism involves androgenic maintenance of certain enzymatic activities, mediated through other hormones such as growth hormone, insulin, and somatostatin (Maiter *et al.*, 1991, Skett, 1988).

This work demonstrates that testosterone plays an inhibitory role in the excretion of conjugated S-ET into urine, suggesting that the disposition of ET conjugates into urine are under the control of the hypothalmic-pituitary axis rather than "imprinted" sex dependent metabolic pathways. The fact that male rats, which typically show little or no conjugates of ET in urine could be induced by castration to show female patterns of conjugates supports this conclusion (Figure 2). The inhibitory effects of testosterone are also seen in ovarectomized female rats given testosterone, where conjugates of ET in urine are minimal.

Whether the action of testosterone is due to direct inhibition of renal conjugation of ET is unknown, but similar observations have been made for other compounds such as ketoprofen (Palylyk, 1994).) Treatment of gonadectomized male and female rats with testosterone led to suppression of urinary excretion of ketoprofen similar to that seen in intact male rats. Another compound, zenerestat also displays sex hormone dependent renal excretion (Tanaka *et al.*. 1992). Renal clearance experiments suggested that the decrease in urinary excretion of zenerestat in female chronic diabetic rats is due to a decrease in active secretion. The increase in the urinary excretion in male rats was probably due to a decrease in testosterone levels caused by the diabetic state.

2.3.3 Effect of androgens on intestinal toxicity of ET

It is believed that females, especially elderly females, suffer from G.I. related side effects of NSAIDs at a much higher frequency than males (Famaey and Paulus, 1992). Whether this is due solely to higher use by females or a fundamental difference in sensitivity to G.I. damage is not clear.

It has been established that increases in G.I. permeability are a precursor to GI damage, and correlates with ulceration (Ford *et al.*, 1995). These permeability changes have also been shown to be as sensitive and reproducible as ulceration as a marker for intestinal toxicity (Ford *et al.*, 1995). In turn, using ^{51}Cr-EDTA as the permeability marker has been shown to be the most sensitive and reproducible method for study of NSAID induced intestinal damage in the rat (Ford *et al.*, 1995). We therefore used this

model to determine if there were underlying sex differences in lower intestinal toxicity of ET.

No significant sex differences in ^{51}Cr-EDTA permeability were noted using the rat model (Figure 3). Gonadectomy and alteration of sex hormones also did not significantly alter intestinal permeabilty, suggesting that lower intestinal permeability to^{51}Cr-EDTA and the biochemical pathways that affect it are not regulated by sex hormones. It may also be speculated that since many of the biochemical mechansims responsible for cytoprotection are similar throughout the GIT, that sex related differences in other areas of the GIT may be minimal as well, however there is no direct evidence for this.

The lack of sex differences in the toxicity of ET are not suprising. The minimal sex related alterations in the kinetics of ET did not appear sufficient to alter the observed toxicity of this compound. As well the intestinal toxicity of ET in the rat is lower than for most other NSAIDs (Melarange *et al.*, 1994, Davies, 1996). It has been speculated that the relative safety of ET is due to its COX II selective nature (O'Neill *et al.*, 1995). This minimal toxicity of ET makes it difficult to detect subtle differences in permeability. This relative safety of ET is also observed in humans using other global assessment measures (Karbowski, 1989).

A similar lack of sex differences in ^{51}Cr-EDTA permeability following oral single doses of ketoprofen were observed in female, male, and ovariectomized rats (Palylyk, 1994). With ketoprofen, the results were somewhat suprising since females have a much lower clearance than males, thereby increasing their exposure to the drug. For both ketoprofen and ET, the lack of differences may have been a result of the dose

used or the use of single dose vs chronic administration. Equally, these findings do not rule out the possibility that sex differences in other toxicities (such as renal) may occur.

Comparison of results obtained in rats here to GI toxicity reported in humans is complicated by a number of factors. Sex differences in human NSAID induced GI toxicity may be due to differences in pharmacokinetics, disease/drug interaction, and prevalence of NSAID use. The higher prevalence of NSAID induced GI toxicity in human females is in part a function of the higher prevalence of NSAID use, most likely due the higher prevalence of rheumatic conditions in females. For both ET and ketoprofen in the rat, there was no evidence to support either a biochemical or pharmacokinetic basis for sex related differences in NSAID induced GI damage, even when in the female the exposure to ketoprofen was much greater than that of male rats (Palylyk, 1994).

Our work has demonstrated that the female rat is a suitable model for the study of both the pharmacokinetics and toxicity of ET. Nevertheless, the minimal differences in pharmacokinetics give the female model no significant advantages over the male rat model. The lack of differences in intestinal toxicity also make both sexes suitable for further study of intestinal toxicity of ET. Since much of the toxicity work with NSAIDS has been performed in males, and almost all previous kinetic work in rats had been done in males, we chose to proceed with further studies in the male rat model.

3. Enantiomeric Ratio

3.1 Background

3.1.1 Review of therapeutic uses of altered enantiomeric ratio

To our knowledge, the only attempt to improve the therapeutic effect of a drug in man through manipulation of the enantiomer ratio, other than administering pure enantiomer, has been with indacrinone, a high ceiling relatively long-acting diuretic (Tobert *et al.* 1981). Both enantiomers of indacrinone possess uricosuric effects, but the diuretic and ototoxic activity resides predominantly in the (-)- enantiomer (Tobert *et al.* 1981, Rybak and Whitworth, 1987). Clinical experience with indacrinone has shown that therapeutic doses of racemic indacrinone show transient uricosuric activity, so that, similar to other diuretics, hyperuricemia occurs due to the diuretic induced volume contraction and consequent fall in glomerular filtration. This diuretic induced hyperuricemia can precipitate gout, and may also be an indicator of future coronary heart disease in those patients.

In a multi-center double blinded, randomized study involving 65 healthy men, Tobert *et al.* (1981) studied the effects of 10 mg of (-)- indacrinone given concomitantly with 0, 10, 20, 40, and 80 mg of (+)- indacrinone on plasma urate and urate clearance (Tobert *et al.* 1981). It was found that enantiomeric ratios (-/+) of 10/0, 10/10, and 10/20 elevated plasma urate by 8% to 16%, 10/40 was approximately

isouremic, and 10/80 lowered plasma urate by 16%. In this study the potential for altered physicochemical or pharmacokinetic properties was not considered.

3.1.2 The effect of altered enantiomeric ratio on physicochemical properties

Unlike the pharmacokinetics and pharmacology of enantiomers, the effect of altering enantiomeric ratio on the physical properties of non-pharmaceutical chiral compounds has been clearly determined. It is well established that the properties of combined enantiomers, especially when it affects the solid state, are unique from those of each enantiomer alone. (Jacques *et al.* 1981). Yet even these changes are infrequently noted in the pharmaceutical literature.

Crystalline state

When equal amounts of enantiomers are present, the product is called a racemate, irrespective of phase (American Chemical Society, 1970). This racemate may be classified into 3 types, based on melting point phase diagrams constructed by altering enantiomeric ratio from 100 % of one enantiomer to 100 % of its antipode (Figure 4).

The first type of racemic solid is the conglomerate (Figure 4A). This is formed when the two enantiomers crystallize separately, forming a two phase physical mixture. The binary phase diagram for the conglomerate yields only one eutectic which corresponds to the melting point of the conglomerate.

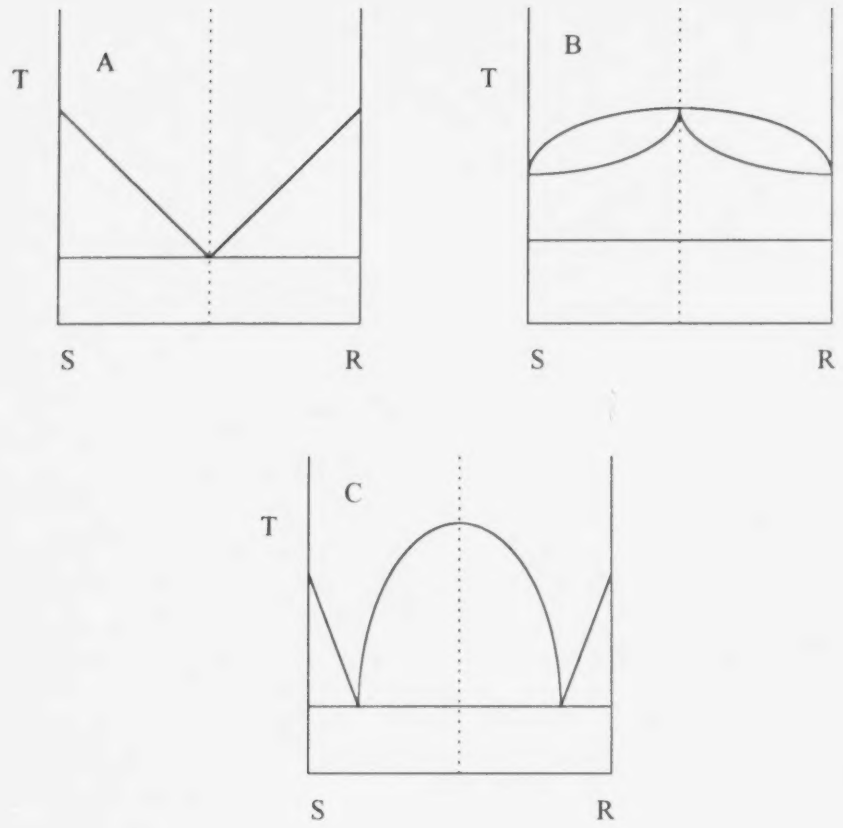

Figure 4. Binary Phase Diagrams illustrating the three fundamental types of crystalline racemates; conglomerate (A), mixed crystals (B), and true racemate (C). T is temperature.

The second situation occurs when the two enantiomers form a continuous series of solid solutions (mixed crystals), the one of which has equimolar enantiomeric composition being designated the racemic solid solution or the pseudoracemate (Figure 4B). For this type of compound, the various crystals may show no change in melting point, a depressed melting point for the racemate, or a maxima for the melting point of the racemate. For example, praziquantel, a drug used in the treatment of the

parasitic disease schistosomasis exhibits pseudoracemate behaviour with a maxima for the racemate (Lim *et al.* 1995).

The most common form of the racemate is the true racemate (Figure 4C), which contains equal amounts of both enantiomers in each individual unit cell of the crystal. These types of addition compounds (compounds in which more than one type of molecule is present in the unit cell of the crystal) are characterized by the formation of two eutectics. When equimolar amounts of each enantiomer are present, only the racemic addition compound is formed. However, if one enantiomer is predominant, the true racemate is formed and the excess of the predominant enantiomer crystallizes as the pure enantiomer. This occurs in the ratio of 2x-1 moles of the predominant enantiomer and 2(1-x) moles of true racemate where x is the mole fraction of the predominant enantiomer (Jacques *et al.* 1981). The melting point of the true racemate may be above the melting point of the enantiomers (as illustrated) if the free energy of the true racemate is less than that of the individual enantiomers, or below, if the free energy of the true racemate is greater than that of the individual enantiomers.

The eutectics in this form of crystalline addition compound are defined by the points where the racemic crystal, the predominant enantiomer crystal, and the liquidus form of both co-exist. The areas distal to the eutectic points bounded by the liquidus curve above and the tie-line below indicate where the racemic crystal has melted and the pure predominant enantiomer exists in the solid form. In this area, the simplified equation of Schroder-Van laar can be used to describe the part of the liquidus curve

between the pure enantiomers and the eutectic in schematic B of Figure 4 (Jacques *et al.* 1981);

$$\ln x = \frac{\Delta H_A^f}{R}\left(\frac{1}{T_A^f} - \frac{1}{T^f}\right) \qquad \textit{Equation 1}$$

where x is the mole fraction of the more abundant enantiomer, ΔH_A^f (cal/mol) and T_A^f (K) are the enthalpy of fusion and melting points of the pure enantiomers respectively, and T^f is the melting point of the true racemate.

In the remaining portion of the curve between the eutectics, bounded by the tie-line below and the liquidus curve above, the predominant enantiomer crystal has melted and pure addition compound remains. This area can be described by the equation of Prigogine-Defay (Jacques *et al.* 1981);

$$\ln 4x(1-x) = \frac{2\Delta H_R^f}{R}\left(\frac{1}{T_R^f} - \frac{1}{T^f}\right) \qquad \textit{Equation 2}$$

where x is the mole fraction of one of the enantiomers, with melting point T^f, (K), T_R^f and ΔH_R^f (cal/mol) are respectively, the melting point and the enthalpy of fusion of the true racemate. From either of these equations, the enthalpies of fusion of the racemate and the pure enantiomer may be obtained from the slope of the lines resulting from rearrangement of the two equations.

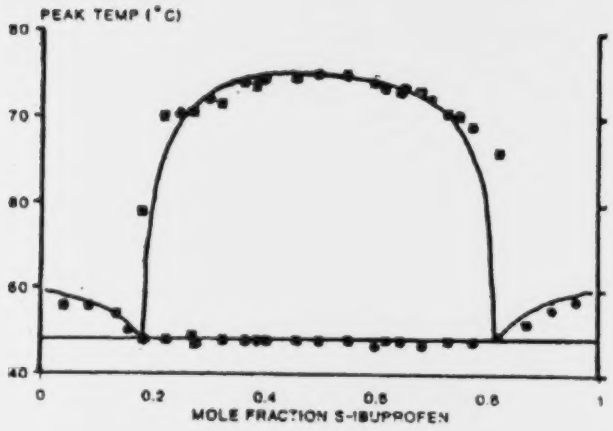

Figure 5. Representative binary phase diagram of ibuprofen

Illustrative of this true racemate phenomena is ibuprofen (Figure 5), which shows the two eutectics in the binary phase diagram (Dwivedi *et al.* 1992; Romero and Rhodes, 1991). The actual observed value for the eutectics is a predominant enantiomer molar fraction of 0.82. There is some discrepancy on the exact composition of the eutectic (0.82 vs 0.90 molar fraction) due to the fact that in one report the authors calculated the eutectic from extrapolation (Romero and Rhodes, 1991) while the others used actual observed values (Dwivedi *et al.* 1992). Both the pure enantiomers of ibuprofen and racemic crystals contained four molecules in the unit crystal. However the racemic occupies the P2(1/c) space group while the pure enantiomer the P2(1). Thus intermolecular forces are different for the two crystals resulting in different melting points (Romero and Rhodes, 1991).

DSC data on true racemates also exhibit interesting properties. Ibuprofen DSC data on compounds of mixed racemic and pure crystal show an endotherm at the eutectic temperature (which defines the tie-line for the binary phase diagram), as well

as an endotherm at a depressed temperature for the racemic or pure enantiomer crystal, depending on which species is predominant (Figure 6).

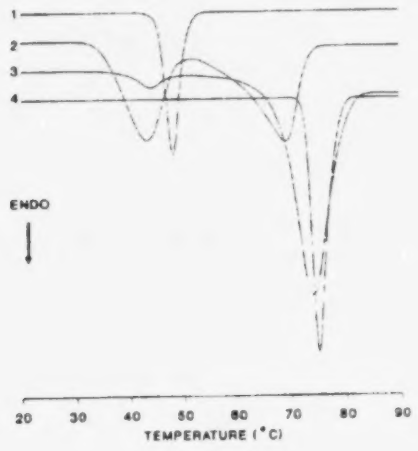

Figure 6. Representative DSC thermograms of ibuprofen compounds of various enantiomeric composition. Lines 1,2,3,4 are 96%, 27.6 % and, 46 % S-ibuprofen, and racemic ibuprofen respectively.

DSC data on true racemates also exhibit interesting properties. Ibuprofen DSC data on compounds of mixed racemic and pure crystal show an endotherm at the eutectic temperature (which defines the tie-line for the binary phase diagram), as well as an endotherm at a depressed temperature for the racemic or pure enantiomer crystal, depending on which species is predominant (Figure 6).

Solutions

The unique properties of various ratios of enantiomers are not limited to the solid phase. The fact that changes in solubility for various ratios of enantiomers occur

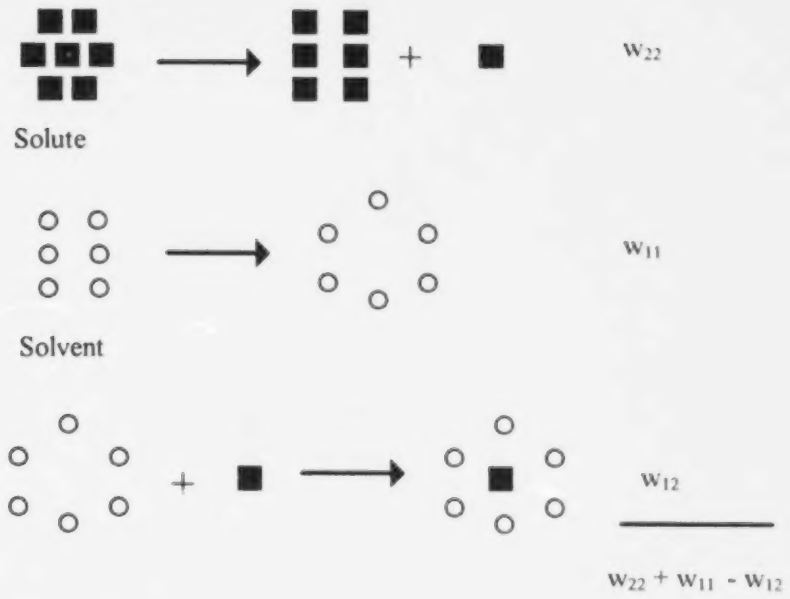

Figure 7. Processes involved in the dissolution of a crystalline solute
1. Energy input for removal of solute molecule from solute array (w22)
2. Energy input for formation of cavity in solvent (w11)
3. Energy release from inclusion of solute in solvent (w12)
Overall energy requirement is summation of these energies

has been used as a tool for the determination of enantiomeric purity for those compounds that are thermally unstable, infusible, or which exhibit complex polymorphism.

The differences in solubility between compounds formed by various ratios of enantiomers can be been explained using the above schematic (Figure 7, Florence and Attwood, 1982). In a simplified sense, the process of forming a solution from an

enatiomeric compound involves two parts. First, individual molecules from solid crystals must be removed, requiring a net input of energy (w_{22}). The stronger the crystal (as reflected in a higher melting point), the more energy that is required to remove a molecule.

Second, cavities in the ordered array of the solvent must be formed to allow solute molecules to be transferred into them (w_{11}). The overall work required to perform this is the addition of these two processes shown in the above diagram. In a non-ideal solution, solute-solvent interaction occurs, and is generally an exothermic process (w_{12}). (Florence and Attwood, 1982).

For true racemates in a non-chiral solvent such as water, where the work required to form the cavity in the solvent and the interaction between solute and solvent is the same for both enantiomers, the solubility simply becomes a function of the stability of the crystalline state, as determined by melting point. Typically for most true racemates the melting points are in the following sequence: racemate > pure enantiomer > eutectic. Although water exhibits unique solute-solvent interactions, it is not possible for these to be stereoselective. As a result, the most stable (usually the racemate) exhibits the lowest solubility, the pure enantiomer, intermediate solubility, and the eutectic the highest solubility. The exact inverse is true for those true racemates whose melting point is below that of the pure enatiomer, such as ketorolac (racemic melting point 156 °C, pure enantiomer 176 °C) (Roy *et al.)* and sobrerol

(racemic melting point 135 ° C, pure enantiomer 156 °C)(1995; Bettinetti *et al.* 1990).

The equation derived from thermodynamic considerations for an ideal solution of a solid in a liquid and which relates melting point (T_m), ambient temperature (T) and the heat of fusion (ΔH_f) is

$$\log x_2 = \frac{\Delta H_f}{2.303R}\left[\frac{T_m - T}{T_m T}\right] \quad \textit{Equation 3}$$

which for non-ideal solutions (which arises from interactions between molecules of solvent and solute) can be rewritten as

$$\log x_2 = \frac{\Delta H_f}{2.303R}\left[\frac{T_m - T}{T_m T}\right] - \log \gamma_2 \quad \textit{Equation 4}$$

where γ_2 is the rational (interaction) coefficient, and is dependent on both solute and solvent properties (Martin *et al.* 1983). A linear relationship between solubility and the inverse of melting point can be plotted from rearrangement of the above equations.

The solubility of weak electrolytes are somewhat more complicated. Most of the NSAIDs are weak electrolytes, with acidic pKa values ranging from 3 to 6. The solubility of a weak electrolytes is dependent on four parameters: 1) degree of ionization, 2) molecular size, 3) interactions of substituent groups with solvent, and 4) crystal properties. (Florence and Attwood, 1982). Factors 2 and 3 are constant for any given drug. Thus the relative ratio of the unionized and ionized species must also be taken into account when considering the solubility of these compounds. However, at any given pH, the linear relationship between solubility and the inverse of melting point

will still hold. In-vivo, except for the time that they reside in the stomach, enantiomers of NSAIDs such as ET (pKa 4.65), exist primarily in the ionized form.

Partition coefficient

Partition coefficient is the relative solubility between any two phases, typically water and oil. It is clear that solubility in an aqueous phase can be altered by enantiomeric ratio. The same is true for octanol solubility (Yalkowsky *et al.* 1983). As a result, the relative partitioning of enantiomers into each phase of a saturated solution may be varied for differing enantiomeric ratios since the relationship between solubility ratio and partition coefficient, although linear through a range of compounds, may deviate from linearity for a single compound thereby altering the partition coefficient for the drug (Yalkowsky *et al.* 1983).

Recent work has attempted to relate partition coefficient to melting point coefficient and aqueous solubility (Yalkowsky and Valvani, 1995; Amidon and Williams, 1982; Chiou and Schmedding, 1981; Yalkowsky, 1981). An equation, based on thermodynamic considerations, has been derived to estimate partition coefficient from these parameters (Yalkowsky and Valvani, 1995).

$$\log sw = -1.00 \log PC - 1.11 \frac{\Delta S_f (MP - 25)}{1.364} + 0.54 \qquad \textit{Equation 5}$$

where sw is water solubility, ΔS_f is the molar entropy of fusion and MP is melting point (°C). For rigid molecules, ΔS_f is equal to 13.5 (Yalkowsky and Valvani, 1995).

3.1.3 Relation of physical properties of enantiomers to biological properties

The influence of enantiomeric composition on partition coefficient of chiral drugs has not been addressed in the literature. Nevertheless, it is clear from the previous equation (Equation 5) that changes in solubility may influence partition coefficient for a saturated solution. As discussed before, the solubility of chiral addition compounds is a function of their enantiomeric composition.

The physical constant partition coefficient provides a surrogate measurement indicative of a compounds ability to move through a biological system. Various drug activities, toxicities, and distribution processes have been sucessfully correlated with partition coefficient (Hansch, 1972). It is now generally accepted that physicochemical properties (e.g., solubility, melting point) play an important role in the disposition of the compound *in vivo*.

Currently there are multiple methods for determining partition coefficient. The most commonly used method employs 1-octanol and water as surrogates for the partitioning of drug into biological membranes. This method is limited however, to compounds that have large partition coefficients (Gobas *et al.* 1988). Often loss of linearity is observed for such compounds due to the fact that membranes and other lipid phases in organisms possess a more definite structure than octanol, and thus differences in phase behaviour occur. Fortunately, ET has a small partition coefficient and hence the octanol water partition method provides a suitable surrogate measure of partitioning.

The importance of partition coefficent becomes more clear as an attempt is made to describe what factors impact upon the disposition of the drug in the body. The relationship between the partition coefficient and the passive rate of diffusion through a membrane is given by a later version of Fick's Law (Martin *et al.* 1983).

$$\frac{dQ}{dt} = \frac{DAK}{h}(c_d - c_r) \qquad \textit{Equation 6}$$

where dQ/dt is the rate of diffusion, D is the diffusion coefficient (a function of the membrane), A is the surface area of the membrane, K is the partition coefficient, h the membrane thickness and c_d and c_r are the concentrations on the donor and recipient side of the membrane respectively.

It is currently a topic of debate whether the movement through a biomembrane can be stereoselective (Heard and Brain, 1995). The stratum corneum, which acts as the rate limiting barrier to percutaneous penetration contains chiral compounds such as keratin and ceramides. However, evidence for intrinsic stereoselectivity in skin permeation has yet to be obtained.

It is clear from the preceding discussion and Fick's law, that for a given enantiomeric ratio, if the partition coefficient can be altered, the rate of diffusion into a tissue can also be changed. Evidence for these phenomenon for NSAIDs comes from a recent work (Roy *et al.* 1995). Racemic ketorolac showed significantly higher (33-200%) permeation rates through both a chiral bio-matrix (cadaver skin) as well as a non-chiral matrix (poly-ethylenevinyl acetate matrix) as compared to the pure enantiomers, which were not significantly different from one another. Although the

authors could not offer an explanation for their findings, from Fick's law it is clear that partition coefficient must have been altered favorably for the racemic over the pure enantiomers due to altered solubility, since the diffusion constant D must be the same for both enantiomers in the non-chiral matrix. Further evidence of this phenomenon is seen with other chiral drugs such as norgestrel, where permeation rates across various human biomembranes is higher for the racemate than for either of the enantiomers as well (Chien and Nair, 1993).

3.2 Materials and methods

3.2.1 Chemicals

Racemic ET, as well as R and S-ET enantiomers were provided by Wyeth-Ayerst Laboratories (New York, NY, USA). Internal standard [(±)-2-(4-benzoylphenyl)butyric acid], was provided by Rhone-Poulenc (Montreal, PQ, Canada). Isopropyl alcohol, ethyl chloroformate, iso-octane, HPLC grade chloroform, HPLC grade water, H_2SO_4, NaOH, and diethyl ether were purchased from BDH chemicals. Methoxyflurane (Metofane) was obtained from Pitman-Moore Ltd (Mississauga, Canada), polyethylene glycol 400 from Fisher Scientific (Edmonton, Canada), heparin (Hepalean) from Organon Teknika (Toronto, Canada). ^{51}Cr-EDTA was purchased from NEN Dupont,Wilmington, Deleware (specific activity 570MCi/mg).

3.2.2 Assays

Analysis of ET enantiomers in biological fluids

Analysis of the enantiomers of ET in plasma was determined in the same manner as in the previous chapter (Section 2.2.2 Analysis of etodolac enantiomers in biological fluids).

Tissue extraction

Modifications of the plasma assay for ET, which included addition of a water wash phase, were used to determine concentrations of drugs in various tissues (Brocks and Jamali, 1991) . Tissue homogenates spiked with IS were transferred to test tubes and acidified using 200 µl of 0.6 M H_2SO_4. Extraction of ET and IS was performed with 6 ml of iso-octane:isopropanol (95:5). Following transfer of the organic layer to clean tubes and addition of 6 mL of HPLC grade water, mixing, and centrifugation, the organic layer was discarded. The aqueous layer was then acidified with 0.6 M H_2SO_4 and 6 mL chloroform was added. The biphasic mixture was mixed and centrifuged and the aqueous layer was removed. The remaining chloroform layer was evaporated to dryness.

3.2.3 Surgical Procedures

Jugular vein cannulation

Catheterization of male rats at the right jugular vein was performed using the methods described in the previous chapter (Section Jugular vein cannulation). Rats were fasted overnight and until 3 h post dose.

Dosing and sample collection

All ET doses in the pharmacokinetic studies were dissolved in polyethylene glycol 400 and administered at 9 am on the day following surgery. Intravenous doses were given into the jugular vein cannula followed by 0.2 ml of 100 unit per ml heparinized saline. Oral doses were administered using a 18 gauge gavage needle. Blood (0.2 ml) was collected from the jugular vein cannula following ET administration. The catheter was flushed with 0.2 ml of 100 U/ml heparin following dosing and after each blood sample collection. Immediately following collection, plasma was separated from blood by centrifugation at 1800g for 3 minutes using a Fisher model 235A microcentrifuge (Fisher Scientific, Edmonton, Canada). All samples were stored at -20°C until analyzed.

3.2.4 Specific Study Designs

ET Compounds with varying enantiomer composition

ET compounds were prepared by dissolving enantiomers in isopropanol and evaporating the solvent. For melting point determinations, compounds were prepared in 5% intervals. For other studies, molar ratios corresponding to the eutectic (3/1 molar ratio), racemate, 5/5, 5/3, and 5/1 (S/R) were also prepared. HPLC analysis of the formed compounds was performed to ensure no degradation or inversion of ET enantiomers occurred during the process.

Melting points

Melting points of the different mixtures of ET were determined using a capillary tube melting point apparatus. Points represent the mean of three measurements. Starting temperature for all melting point measurements was 60° C., at a rate of 5° C through the melting points.

Thermal studies

Compounds composed of S-ET, Racemic ET, and a 75/25 ratio (the eutectic ratio) of ET enantiomers were placed in individual aluminum DSC pans and sealed. Samples were heated to 60° C and then heated at a rate of 5° C through the melting points in a Seiko SSC/5200 differential scanning calorimeter (Plymouth Meeting, Pennsylvania, USA). Energy input as a function of temperature were recorded.

Solubility studies

Racemic, eutectic (72/25 enantiomeric ratio) and S-ET were placed in 1 ml of phosphate buffer (pH 6.97) in excess of maximum solubility (n = 3 for each compound). Samples were stirred at 1000 hz for 24 h after which 100 µl samples were drawn from each tube at 8 and 24 h to ensure that maximum solubility had been reached. Each sample was filtered through a 0.2 µm nylon filter unit (Phenomenex, Torrance, California) attached to a 1ml syringe to ensure that no ET particulate was measured. Fifty µl of this filtered sample was then placed in clean dry test tubes. IS

was added to each sample and evaporated to dryness. Derivatization and extraction were performed and aliqouts were injected into the HPLC system.

Ultraviolet spectrum

ET compounds with S/R enantiomeric ratios of 5/5, 5/3, 5/1 and 5/0 were dissolved in various solutions buffered to pH 2.35, 5.36, 7.6, 10.19, and 12.04. The wavelength of maximum absorbance (λ_{max}) and the maximum absorbance were measured for each compound (n =3) over an range from 200 to 400 nm.

Partition coefficient

Partition coefficient was determined by placing 100 μg of the racemate, the eutectic (75/25 ratio of enantiomers), and the S-ET compounds in test tubes containing 1 ml of octanol and 1 ml of aqueous buffer solution adjusted to pH 1.95 (KCl/HCl buffer), 6.06, 6.97, and 7.90 (potassium phosphate, NaOH buffer), and pH 10.54 (KCl/boric acid/NaOH buffer). The test tubes were shaken at 1000 Hz at 25°C for 72 h after which 200 μl samples were taken from both phases of each beaker and assayed. All experiments were performed in triplicate.

Pharmacokinetic studies

The influence of enantiomer ratio on the plasma-time course of ET

Jugular vein cannulation was performed under methoxyflurane anesthesia on 16 male Sprague Dawley rats. The animals were allowed to recover overnight. Four

groups of 4 rats were orally dosed with 10mg/kg S-ET and varying amounts of R-ET to yield S/R dosing ratios of 5/5, 5/3, 5/1, and 5/0. Blood samples were withdrawn from the cannula at 0, 0.33, 0.66 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 h after drug administration.

Tissue distribution

Sixteen animals were dosed intra-peritoneally with one of 4 different solutions containing equal amounts of the active S-ET (5 mg/Kg) and varying amounts of R-ET to yield S/R ratios of 5/5, 5/3, 5/1, and S-Et alone (n=4 for each group). All solutions were dissolved in polyethylene glycol 400. Animals were sacrificed and stomach, duodenum, heart, liver, kidneys, and plasma were collected Four h post-dosing, rinsed (with the exception of plasma) with phosphate buffer to remove excess blood, weighed, and immediately frozen at -20 C until analyzed. Tissues were homogenized using Potter-Elvehjem glass tissue grinders driven by a T-line Laboratory stirrer placed in an ice bath to ensure no degradation of ET (Talboys Engineering, Montrose PA). HPLC grade water was added when required and final volumes of each tissue were recorded. Aliquots were then taken for HPLC analysis.

Plasma protein binding

Protein binding studies were performed via an equilibrium dialysis method. A Spectrum protein binding apparatus with Sigma Diagnostic dialysis sacs separating the chambers (molecular weight cut off = 12,000 daltons) was placed in a water bath of 37°C for 6 h. One side of each cell was filled with 1 mL of pooled human plasma, while the other contained 1 mL of isotonic Sorenson's phosphate buffer (pH 7.4)

spiked with 50 ug/mL of S-ET and varying amounts of R-ET to yield S/R ratios of 5/5, 5/3, 5/1, and 5/0.

Intestinal toxicity

Time course

Sprague Dawley rats were orally dosed with 20 mg/kg racemic ET, 10mg/kg R-ET and 10 mg/kg S-ET (n=4 for each dose and each time point). At times corresponding to 0, 1, 2, 3, 4, 6, 8, 12, 24, and 48 h post ET dosing, 0.5 ml of an aqueous solution containing 10 μCi/ml of ^{51}Cr-EDTA was administered using an 18 guage 5 cm curved feeding needle (Harvard Apparatus), attached to a 1 ml syringe. Urine was collected from 0 to 8 h following ^{51}Cr-EDTA administration and assayed for activity as previously described (page 27, Toxicity studies).

Pharmacokinetic / pharmacodynamic link modeling

Data from the racemate (20 mg/kg) and S-ET (10 mg/kg) in the pharmacokinetic study in the previous section (Pharmacokinetic studies) were used with data from the racemate (20mg/kg) and S-ET (10mg/kg) permeability time course (Intestinal toxicity section) to determine if concentrations of S-ET in plasma could be correlated to changes in permeability. A pharmacokinetic pharmacodynamic link model similar to that proposed by Holford and Sheiner (1981) was used to relate effect compartment concentrations and changes in intestinal permeability (Vakily, 1996).

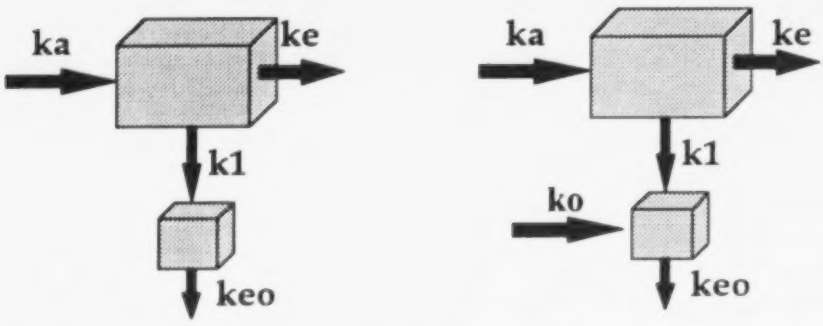

Figure 8. Typical Holford and Sheiner pharmacokinetic-pharmacodynamic link model (left) and a new model (Vakily, 1996, right) which incorporates direct input into the effect compartment

This model utilized a supplemental zero order input directly into the effect compartment to more closely mimic the direct exposure of the gut (the effect compartment) to drug absorption. The model was also suitable for the continuous enterohepatic circulation and subsequent reexposure of the gut to ET (Figure 8, Vakily, 1996). The effect compartment concentrations were then fit to both a sigmoid E_{max} model and a linear effect model using unweighted regression analysis (PCNONLIN 4.1, SCI, Lexington, Kentucky).

Ratio study

Rats were divided into 4 groups of 4 and assigned to one of the following groups: 5/5, 5/3, 5/1, 5/0, where the numerator and the denominator represent the amount in mg/kg of S-ET and R-ET dosed respectively. The corresponding ratios of ET were orally administered to each of the groups at 9 am. Four h post ET dosing, 0.5 ml of an aqueous solution containing 10 μci/ml of ^{51}Cr-EDTA was administered

orally using an 18 gauge 5 cm curved feeding needle (Harvard Apparatus), attached to a 1 ml syringe. Urine was collected from 0 to 8 h following ^{51}Cr-EDTA administration and γ emissions were recorded as previously described. The groups were then crossed over in a randomized block design with a one week washout period between dosing for 4 consecutive weeks so that each rat received all 4 ratios of the drug.

Data analysis

Pharmacokinetic indices

AUC_{0-t} was measured by the linear trapezoidal method. $AUC_{0-\infty}$ was calculated by $AUC_{0-\infty} = AUC_{0-t} + C_t / \beta$, where t was the last sampling time and C_t the last measured concentration. The rate constant of the log-linear terminal portion (β) was estimated using fitted non-linear regression. The oral clearance (CL/F) was calculated using the enantiomeric dose divided by the respective $AUC_{0-\infty}$. The apparent volume of distribution was calculated as $Vd/F = D / AUC_{0-\infty} * \beta$ where F is the fraction of the dose absorbed.

Statistical analysis

All statisitical analyses performed were parametric in nature at an $\alpha = 0.05$ level of significance. Comparison of two means employed standard Student's t-test. Repeated measures t-tests were used for comparison of two means when baseline values had been subtracted. Comparison of means greater than two utilized one-way ANOVA with Scheffe's post-hoc analysis. Treatment order effect in multiple group

studies was controlled by placing equivalent numbers of samples in each treatment period. Studies employing two variables were analyzed using two way ANOVA. Error bars on all figures indicate standard deviation.

3.3 Results and Discussion

3.3.1 Physicochemical properties

Melting points

In the solid phase, the molecular chirality associated with an optically active compound is a product of its unique crystallography (Brittain, 1990). Racemic materials can be classified by their melting point phase diagrams, which provide an understanding of the nature of the crystals that are formed when the two enantiomers are brought together in various ratios.

Formation of the ET enantiomers into the compounds of various ratios occurred without degradation or chiral inversion. The melting point binary phase diagram (Figure 9) suggests that 3 unique crystals are present in the system; S-ET, R-ET, and racemic-ET. Two distinct eutectics are present in the diagram, ruling out the possibility of a conglomerate. The three sharp inflections in the binary phase curve are also atypical of formation of a continous series of solid solutions, ruling out formation of a pseudoracemate. The higher melting point of the racemate indicates that the preferred state for ET is where the two enantiomers coexist in the unit cell. The most likely crystal structure to which most racemic cystals belong is the P 2_1/C, a

member of the monoclinic system (Jacques *et al.*. 1981), in which, the cell contains two of each enantiomer related by a center of symmetry and a binary screw axis. Racemic-ET is known to form anhydrous orthorhombic crystals belonging to the space group Pbca with unit cell dimensions of a = 8.602, b = 18.586, c = 19.055 Angstroms (Humber *et al.* 1986). The crystal structure of the pure enantiomers of ET has not been reported, but due to their optical activity, must contain a hemihedral facet on the crystal (Brittain, 1990). This limits their crystallography to 11 of 32 crystal classes and 66 space groups. The eutectics of ET compounds (the points where the racemic crystal, the predominant enantiomer crystal, and the liquidus form of both co-exist) formed at a molar fraction ratio of 0.75/0.25 of either enantiomer.

It is interesting to note that each racemic addition compound will exhibit unique melting point properties, depending on its chemical structure and crystal packing. Ketorolac, another NSAID with a chemical structure unique from the 2-APAs, also exhibits true racemate characteristics, but where the melting point of the racemate is lower than that of the optically pure enantiomers (Roy *et al.*, 1995). Thermal studies

The results from DSC graphically demonstrate the physical nature of ET compounds (Figure 10). The true racemate is formed from addition of a one to o' molar ratio of S and R-ET, and is present in DSC as a single endotherm (Figure 10A). For ET, the racemic crystalline addition compound is the most stable form, and exhibits the highest melting point in the binary phase diagram (Figure 9), even higher than that of the constitutive pure enantiomers (Figure 10B). The eutectic, formed by depression of the melting points of the two crystal forms present also appears as a

single point, but is actually a mixture of the depressed racemic crystal and enantiomeric crystal (Figure 10C). The areas distal to the eutectic points bounded by the liquidus curve above and the tie-line below display two peaks, one where the racemic crystal is melting (144.4 ° C) and the other where the pure predominant enantiomer exists in the solid form (135.2 °C) (Figure 10D). The differences in melting point observed between the DSC and the binary phase diagram may be explained by the point at which the melt was recorded (at the start of the melt for the binary phase diagram, and in the middle for DSC) as well as the higher level of imprecision inherent in the melting point apparatus. The same two peak phenomena would be seen for DSC obtained proximal to the eutectic points, where the pure enantiomer is melting and the racemic crystal exists in the solid form.

Various equations exist for the description of the areas of the binary phase diagram. The one that describes the area proximal to the eutectics on the binary phase diagram is known as the equation of Prigogine-Defay (Equation 2 ,Jacques *et al.*)

$$\ln 4x(1-x) = \frac{2\Delta H_R^f}{R}\left(\frac{1}{T_R^f} - \frac{1}{T^f}\right)$$

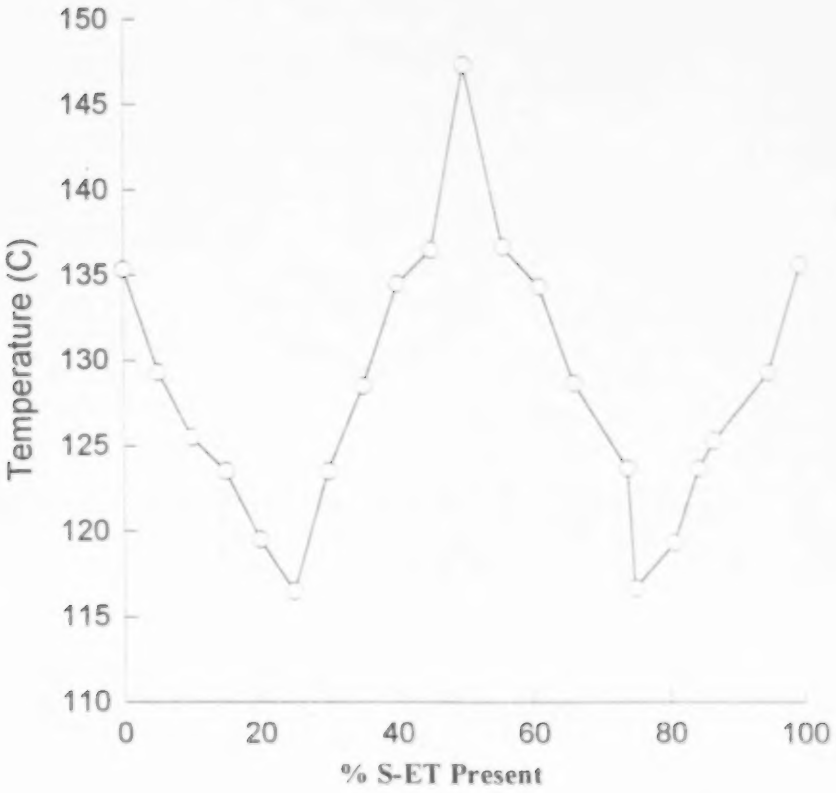

Figure 9. Binary phase diagram of ET compounds (n = 3 at each point)

where x is the mole fraction of one of the enantiomers, T^f, (°K), T_R^f and ΔH_R^f (cal/mol) are respectively, the melting point of enantiomers, the melting point of the racemate, and the enthalpy of fusion of the true racemate. Linearity of the natural log of one minus the mole fraction present vs. the inverse of melting point is definitive evidence of formation of a crystalline addition compound (Figure 11, r = 0.993).

Solubility

Solubility of a compound is an important consideration in the pharmaceutical sciences (Martin *et al.*, 1983). Solubility may be a factor in the stability of a parenteral formulation. It can be a rate limiting step in the dissolution and absorption or an oral dosage formulation. Solubility may also be a rate limiting factor for other dosage forms, such as transdermal delivery. For chiral compounds, differences in solubility are also an important factor in the resolution of enantiomers (Brittain, 1990).

The properties of the three ET compounds at the inflection points of the binary melting point phase diagram (true racemate eutectic, and pure enantiomer) showed

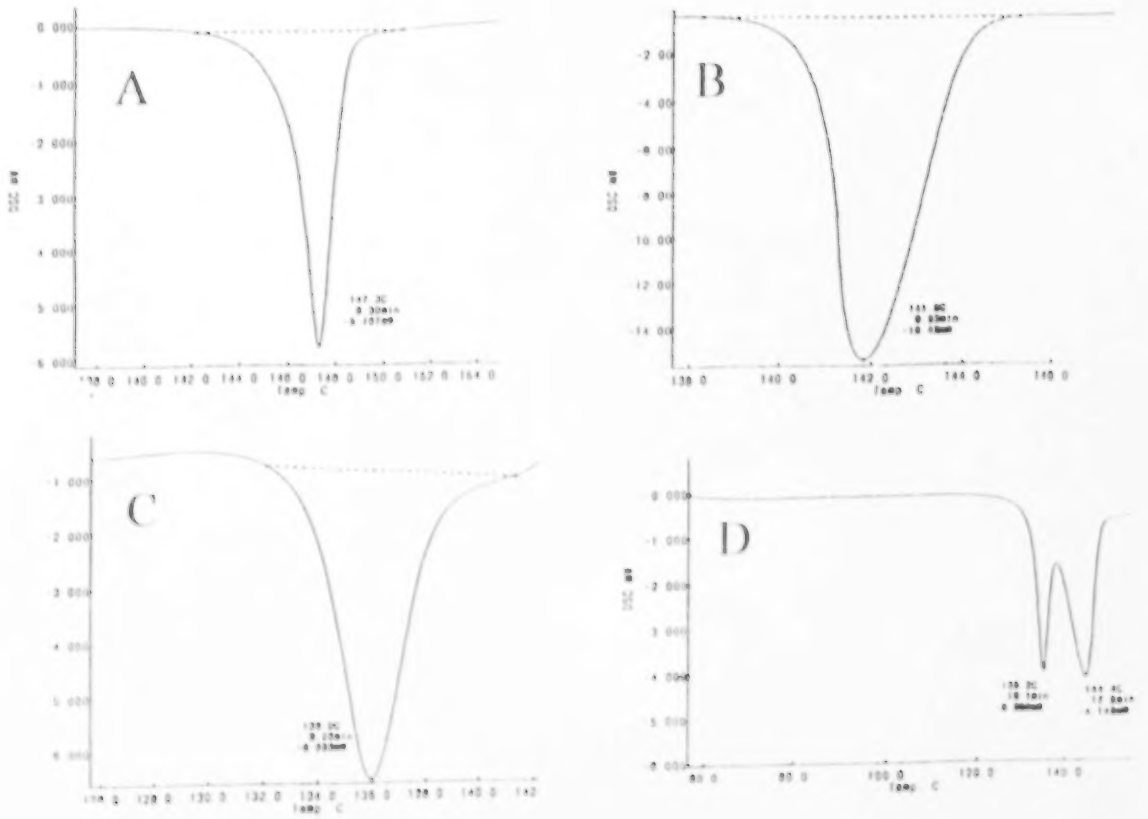

Figure 10. Representative DSC of racemic ET (upper left), S-ET (upper right), eutectic ET (lower left) and a 87% R and 13 % S compound of ET

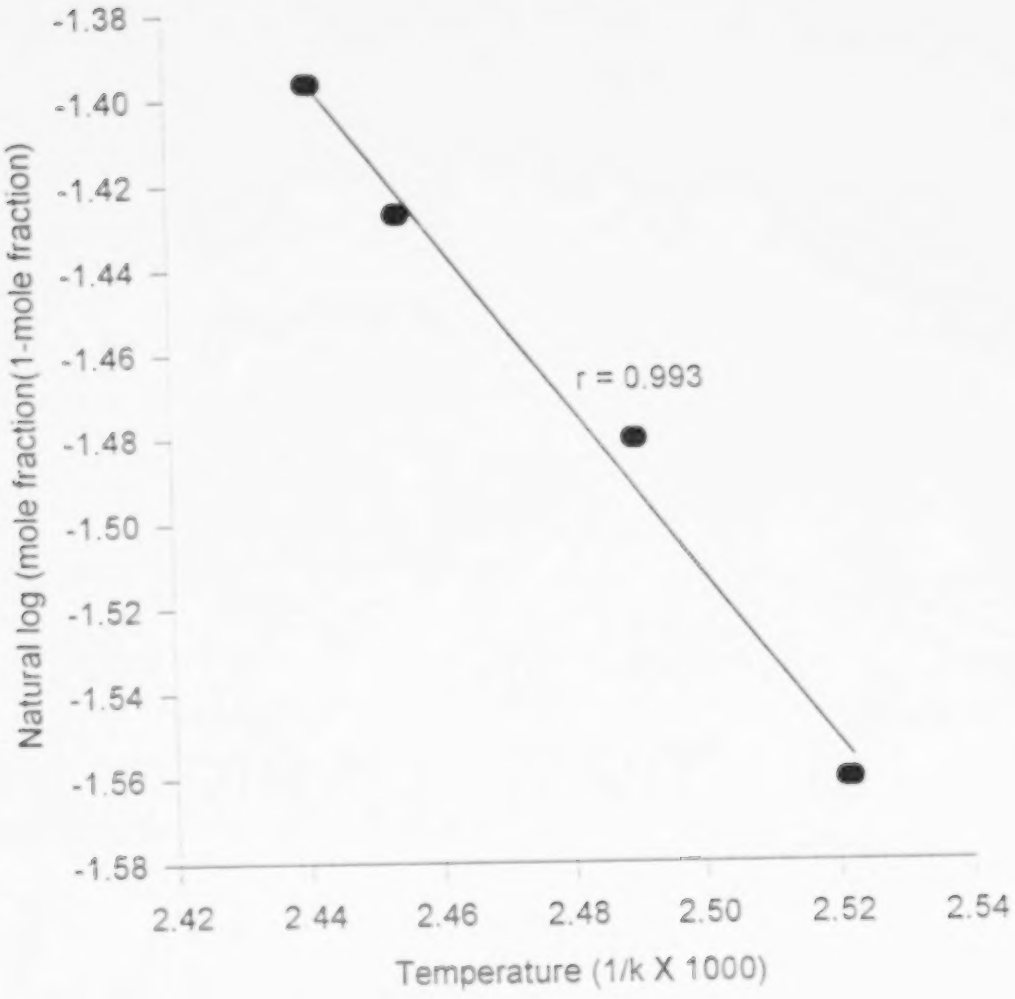

Figure 11. Linear relationship of natural log of molar fraction (1-molar fraction) of S-ET vs inverse of absolute melting point (°K) of S-ET, indicative of formation of a crystalline addition compound (N = 12).*

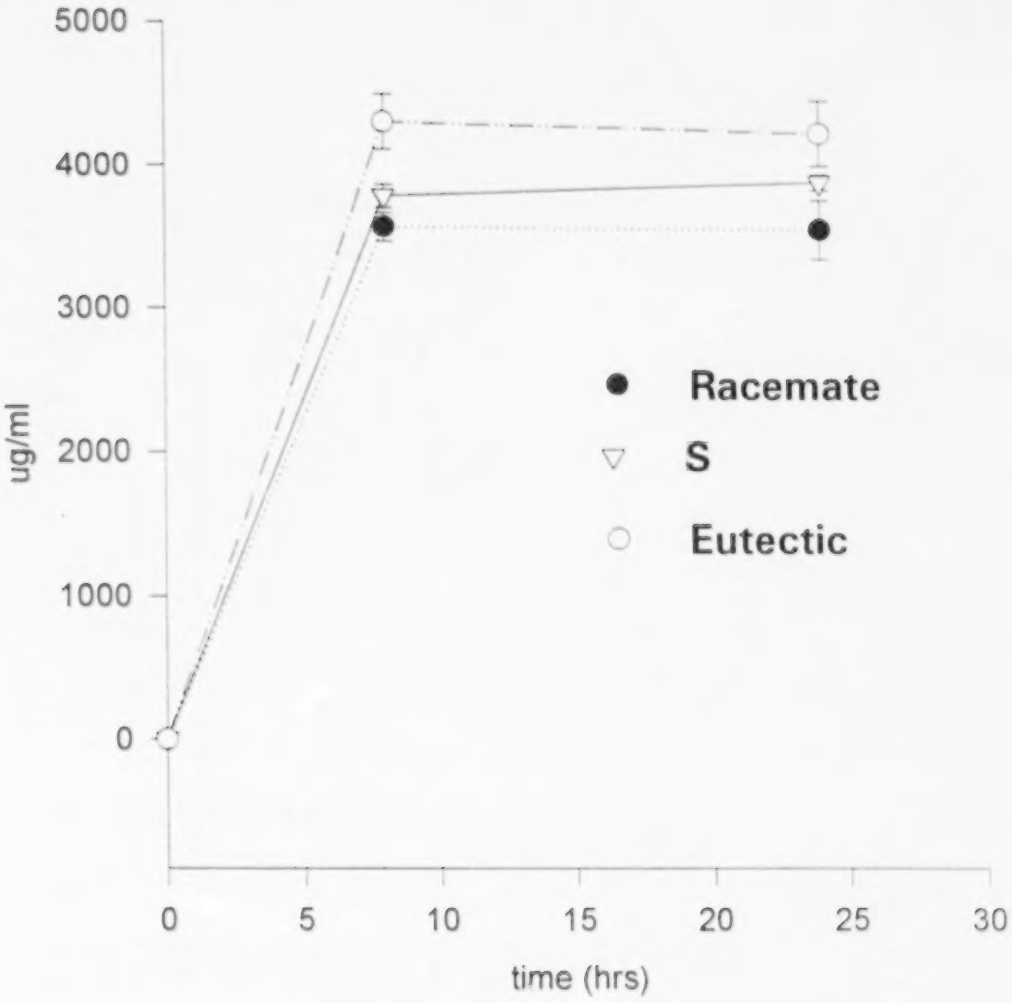

Figure 12. Solubility of true racemate, S and eutectic ET compounds at pH 7.0 (n=3 for each (all 3 compounds significantly different from each other at 8 and 24 hours).

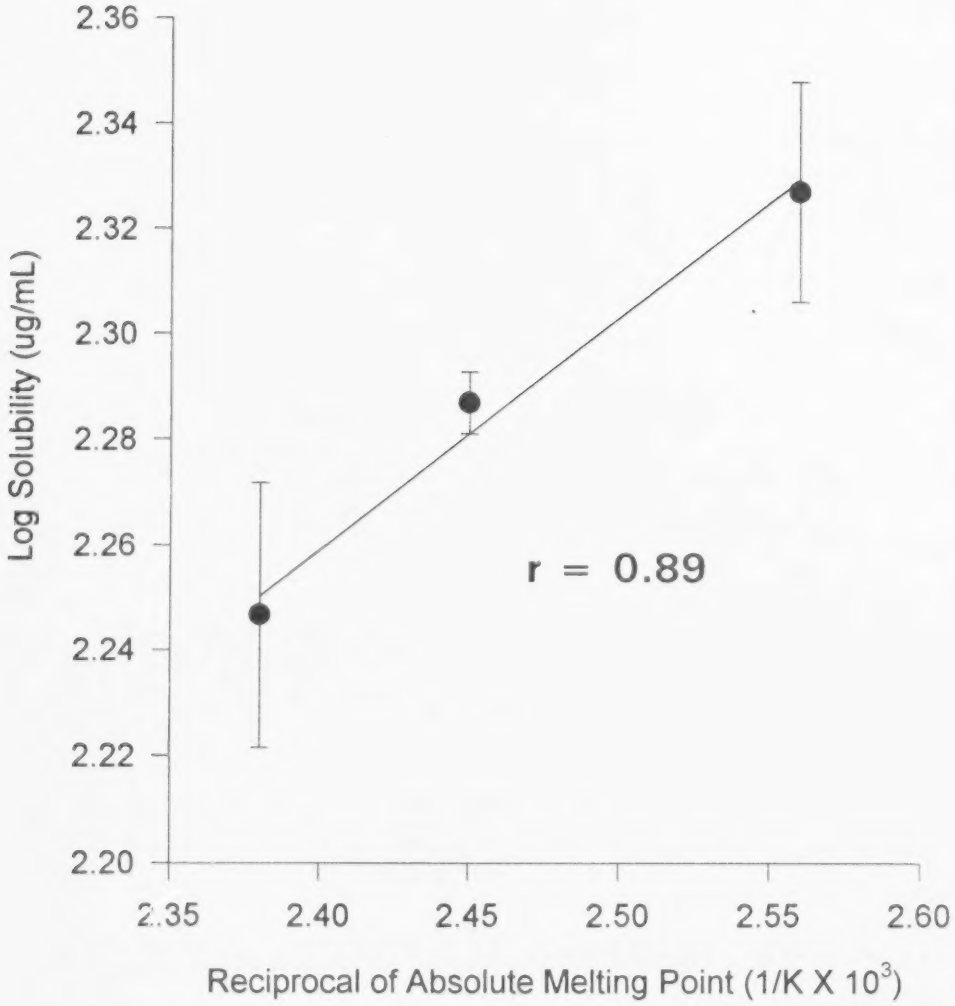

Figure 13. Linear relationship between inverse of melting point (K) of true racemate, eutectic, and S-ET vs solubility at pH 7.0, indicative of an ideal solution formation (n =3 at each point)

significantly different solubilities in aqueous solution at neutral pH (figure12, $p < 0.01$). As expected from thermodynamic theory, solubility of racemic, S and eutectic ET were a function of their respective crystalline state stability (as determined by melting point). The most stable racemic crystal exhibited the lowest solubility, the pure enantiomer, intermediate solubility, and the eutectic the highest solubility.

The relationships between compounds formed of various enantiomeric ratios vary between various NSAIDs, as a function of the underlying crystalline state. Many NSAIDs, such as ibuprofen (Dwivedi *et al.*, 1992) and flurbiprofen (unpublished findings on file) of the 2-APA class exhibit the same pattern of melting as ET. However, in some cases, such as with ibuprofen, greater differences in melting points between enantiomer and racemate yield greater differences in solubility. The opposite pattern of melting and solubility to that of ET has also been observed in those true racemates whose melting points are below that of the pure enantiomer, such as ketorolac (Roy *et al.*, 1995).

Historically, the differences in solubility between enantiomers and racemate have been utilized for enantiomeric separation of conglomerates (Brittain, 1990, Jacques *et al.*, 1981). In the case of the conglomerate, separation may be carried out by a two part method known as resolution by entrainment. In the first step, a solution is prepared which has a slight excess of one enantiomer. Crystallization is induced by addition of a seed crystal, so that the desired enantiomer is obtained as the solid, and the mother liquor is enriched in the other enantiomer. In the second step, the other enantiomer is obtained.

In the case of a racemic addition compound, such as ET or others, resolution by entrainment is not possible. In these cases however, resolution may still be accomplished by derivatization with an optically pure reagent to form two diasteromers. Resolution by entrainment can then be carried out based on the binary phase diagram for the mixture of the two resultant diastereomers. Following resolution, the diastereomers may be cleaved, yielding the optically pure enantiomers (Brittain, 1990).

Although the thermodynamics of crystalline addition compounds are well understood, there has been little attempt in the pharmaceutical industry to capitalize on the unique solubilities of the various ratios of enantiomers, as evidenced by the paucity of literature dealing with this issue. One such application may be the use of the eutectic of chiral compounds to enhance solubility for transdermal drug delivery. In one study, the authors noted that S-ketorolac in racemic form and S-ketorolac alone had different skin flux capacities in human cadaver skin, citing their different melting points and solubilities as the cause (Roy *et al.*, 1995). However, no attempt has been made to utilize a eutectic compound to further increase the solubility of ketorolac.

The effect of ratio specific solvent-solute interaction of ET enantiomers in solution was also determined from the equation derived from thermodynamic considerations for a non-ideal solution (Equation 4) which arises from interactions between molecules of solvent and solute of a solid in a liquid which relates melting point (T_m), ambient temperature (T) and the heat of fusion (ΔH_f):

$$\log x_2 = \frac{\Delta H_f}{2.303R}\left[\frac{T_m - T}{T_m T}\right] - \log \gamma_2$$

where γ_2 is the rational coefficient, and is dependent on both solute and solvent properties (Martin *et al.* 1983). The linear relationship between solubility and the inverse of melting point predicted from rearrangement of the above equation was observed (Figure 13). The relative linearity (r=0.89) of the relationship indicates that no ratio specific interactions (solute-solute or solute-solvent) other than those arising from differences in crystalline state occur at neutral pH in aqueous solution (Florence and Attwood, 1982).

Ultraviolet spectrum

Evidence of the lack of solute-solute interactions other than that of the non-ideal solution equation above is provided by data on the ultraviolet characterstics of ET compounds (Figure 14). No significant changes in absorbance/μg or absorbance maxima of ET compounds were noted over a range of pH indicating that the compounds were stable throughout the pH ranges used in this work, and that ratio specific solute-solvent interactions affecting the extended conjugation of ET did not occur.

A substantially lower absorbance maximum was seen for all ET compounds at pH 2. This observation may be explained by dimerization typical at low pH for compounds containing the carboxylic acid moiety (Martin *et al.*, 1983). Proof for this interaction is typically obtained by IR analysis, with the appearance of the dimer stretch band at approximately 1710 cm^{-1} at high

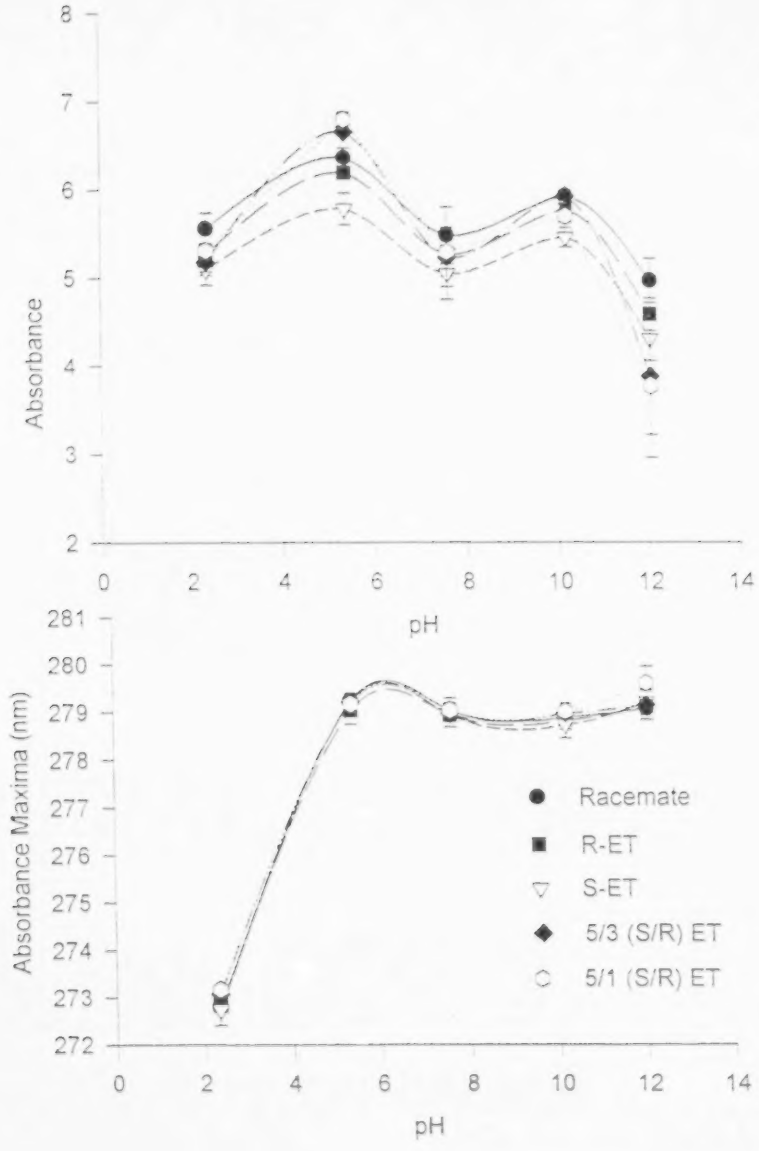

Figure 14. Characteristics of ultraviolet spectrum of various ratios of ET enantiomers (n =3 for each compound at each pH).

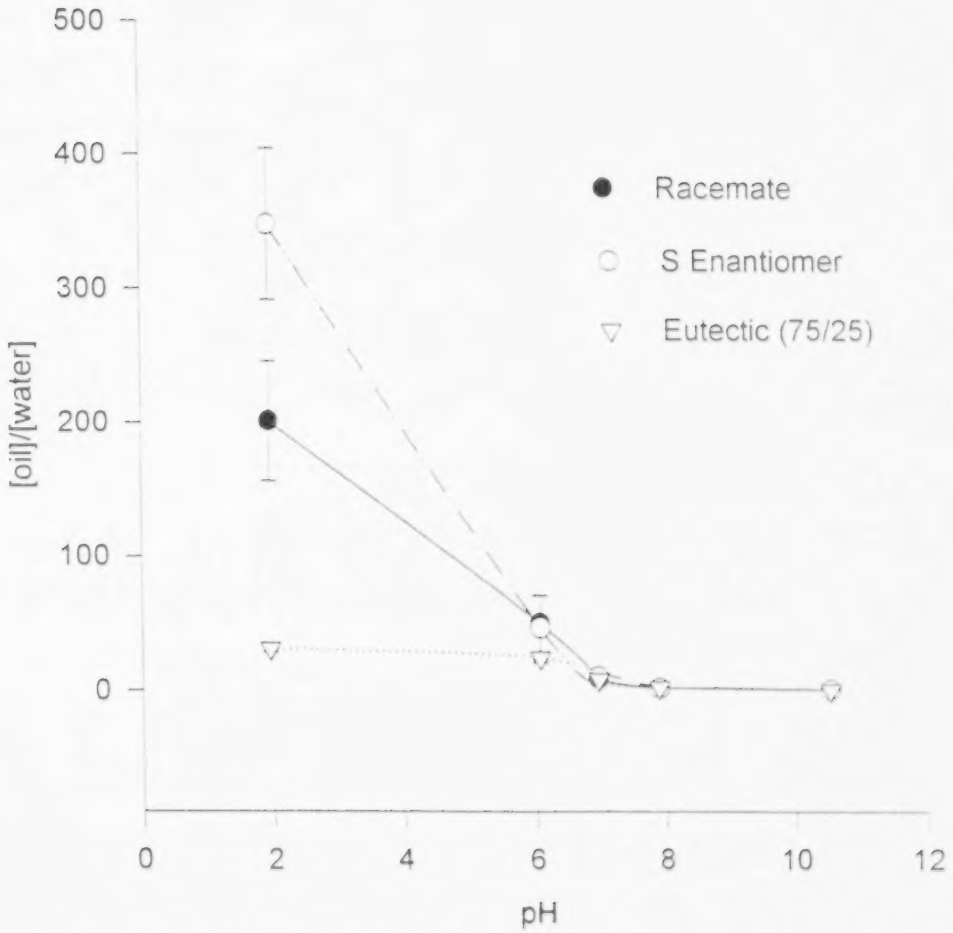

Figure 15. Octanol/water Partitioning of true racemate, eutectic, and S etodolac in a non-saturated state at various pH.

concentrations and disappearance of this band at lower concentrations. However, IR cells suitable for aqueous solutions were unavailable.

Partition coefficient

The concentration of drug in a membrane (C_m) can be given by $C_m = K_p C_d = J^* h/D_m$, where K_p, D_m, and h are the partition coefficient, diffusion coefficient, and thickness of the membrane respectively. For a racemic addition compound r, and an optically pure enantiomer e, the activity coefficient (γ), entropy of fusion (ΔS), and diffusion coefficient (D) are the same for both racemate and pure enantiomer after melting (Brittain *et al.*, 1990). From the previous section, it is clear that the solubility of ET can be related to its melting point. In this case the flux ratios of two compounds differing only by melting point may be given by $Jr/J_e = C_r/C_e = e^{-A/Tmr-Tme)B}$ where the constants A and B are equal to $\Delta S/R$ and γ respectively, and mr and me are the melting points of the racemate and enantiomer. Since partition coefficient is a ratio of the solubilities in two different solvents, the melting point terms cancel. As a result, no differences in the partitioning behaviour between any two ratios of ET should exist (Wearley *et al.*, 1993).

The partitioning of ET compounds into octanol and water in non-saturated conditions showed significant differences as a function of pH (Figure 15). Only at low pH were significant differences in the partitioning of the three compounds observed. Again, this is probably due to formation of unique dimers for each ratio in the acidic conditions, yielding unique physicochemical properties. The differences disappear as the pH of the medium is increased and the molecules of ET disassociate in solution, as expected from the preceding discussion. The

relative decrease in partitioning seen as function of increasing pH is common for weakly acidic compounds due to the increasing degree of ionization in the aqueous solution. (Florence and Attwood, 1982).

The relevance of this pH dependent alteration of partitioning due to altered enantiomer ratio is probably minimal, except perhaps in the stomach, where the pH of the lumen is well below the pKa of ET. For ET, the potential for altered absorption from saturated solutions of ET in the gut is possible, but its significance is probably minimal due to already rapid absorption.

3.3.2 Pharmacokinetics

Previous work by Brocks *et al.* has determined much of the stereoselective disposition of ET in rats (Brocks and Jamali, 1990, Brocks and Jamali, 1991). Following administration of racemic ET, plasma concentrations of the less active R-ET are much greater than those of active S-ET. Significantly greater $t_{1/2}$, CL, and Vd_{ss}, and lower AUC, for S- than for R-ET are observed. Secondary peaks indicative of extensive enterohepatic recirculation were present in plasma time courses of S-ET, and to some degree for R-ET. The differences between enantiomers were attributed to a greater extent of plasma protein binding of R-ET, and to preferential conjugation and biliary excretion of S- ET. Urine was a minor route of elimination in the male rat. No evidence of a pharmacokinetic interaction or inversion between the enantiomers was observed after iv dosing of 5 mg/kg. It was concluded that the rat may be a suitable pharmacokinetic model for the study of stereoselective pharmacokinetics of ET because the results closely paralleled those of ET in man.

To date, linearity of dosing with ET had not been verified for individual enantiomers of ET (Brocks and Jamali, 1994). In this study, dose linearity of R-ET, the predominant enantiomer in plasma was observed with a fixed dose of S-ET and increasing doses of R-ET (Figure 16). The values reported here for clearance, elimination rate, and volume of distribution agree with those reported earlier with single enantiomer and racemic administration of ET at lower doses (Brocks and Jamali, 1990). These results confirm earlier findings of the lack of pharmacokinetic enantiomeric interaction occurring between ET enantiomers seen at lower doses (Brocks and Jamali, 1990). It also confirms that the plasma pharmacokinetics of R-ET remain linear in the rat even at the higher doses of up to 20 mg/Kg required for toxicological work.

The pharmacokinetics of S-ET were complicated and variable, due to enterohepatic circulation. For S-ET, half life appeared extremely long and variable, and as other authors have noted, is a highly variable parameter (Brocks, 1993). Hence, we were not able to determine any differences in the half life of S-ET for any of the ratios tested ($p = 0.354$). Significant differences in the S-ET AUC betweeen the various ratios of ET compounds given were noted (between pure S enantiomer and the S enantiomer from racemate). However, these differences were not significant after correction for animal weight and total dose given, as indicated by the lack of significance in the clearance between groups for S-ET ($p = 0.187$). Similarly, no differences were noted for the volume of distribution term for S-ET ($p = 0.993$) following dosing with the various enantiomer ratios.

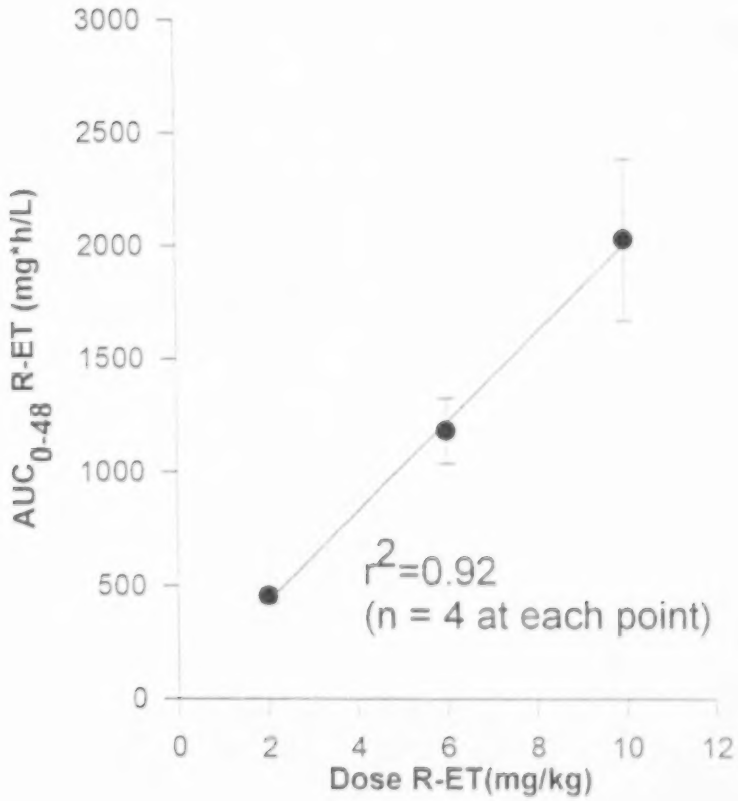

Figure 16. Dose vs. AUC relationships of R following dosing with 10 mg kg of S-ET (n=4 at each time point)

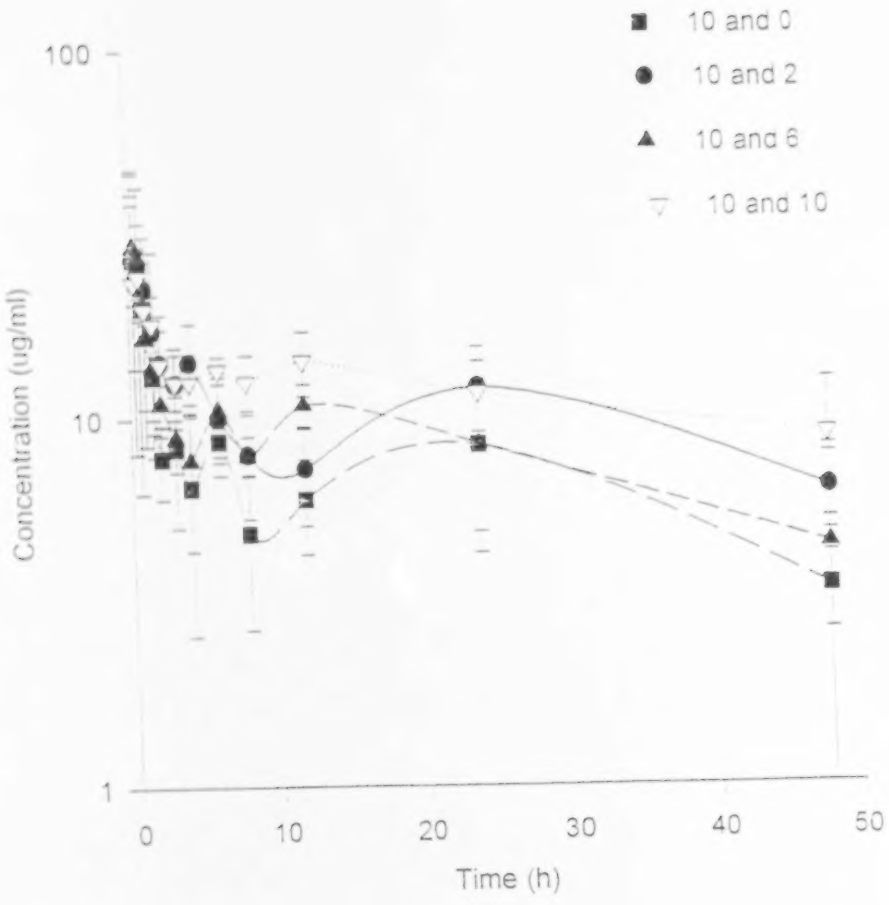

Figure 17. S-ET concentration time relationships after oral dosing with 10 mg kg of S-ET and 0,2,6, and 10 mg/kg of S-ET (n = 4 at each point)

Table 6 Pharmacokinetic parameters (0-48 h) following oral dosing with 10mg kg of S and10, 6, 2, and 0 mg Kg of R-ET

	5 and 5		5 and 3		5 and 1		5 and 0	
	S	R	S	R	S	R	S	R
AUC_t (μg*h/mL)	571 ± 106	2080 ± 374	437 ± 28	1182 ± 145	475 ± 95	454 ± 32	328 ± 75[a]	
Cmax (μg/ml)	35.9 ± 18.7	125 ± 51.6	31.9 ± 7.6	92.56 ± 6.62	31.4 ± 16.4	36.2 ± 11.7	31.2 ± 11.3	
Tmax (h)	1.99 ± 2.68	0.87 ± 0.50	0.42 ± 0.17	0.58 ± 0.32	0.66 ± 0.27	0.99 ± 0.59	0.58 ± 0.17	
$t_{1/2}$ (h)	64 ± 36	22 ± 5.5	43 ± 53	19.3 ± 3.64	32.9 ± 17.6	20.66 ± 2.70	22.6 ± 10.5	
V_d/F (ml)	239 ± 35	40.4 ± 30.8	224 ± 83	58.2 ± 18.8	238 ± 122	41.9 ± 2.95	229 ± 78	

[a] Significantly different than racemic S-ET AUC

C_{max} and T_{max} were also not significantly altered between groups for S-ET (p = 0.96 and 0.37 respectively). R-ET T_{max} was also not altered (p=0.49).

Protein Binding

The unbound fraction of S-ET as a function of enantiomer ratioin pooled human plasma was similar to that seen by previous authors using similar concentrations of S-ET given as the racemate. (Brocks and Jamali, 1991). Although there were no differences between the 4 different ratios of ET enantiomers when compared by ANOVA (p =0.46), there did appear to be a trend to a larger unbound fraction of S-ET with increasing amounts of R-ET (Figure 18, r = 0.48, p =0.096). These findings of increasing unbound fraction of ET enantiomers with increased total drug concentration are consistent with earlier reports utilizing the racemate and non-stereospecific assays (Cayen *et al.*, 1981, Ferdinandi *et al.*, 1986) as well as those utilizing the racemate and reporting free fraction of both enantiomers (Brocks and Jamali, 1991).

The protein binding of ET enantiomers to human serum albumin has been studied in detail (Muller *et al.*, 1992). Free fractions of both S and R were shown to increase as the concentration of each respective enantiomer was increased. Utilizing flourescent site specific markers of human serum albumin, it has been found that both R and S ET bind to site I on albumin, while only S is strongly bound to site II. However, no attempt had been made to determine if increasing concentrations of one ET enantiomer could cause displacement of the other (Muller *et al.*, 1992).

Similar complex binding charactersitics and enantiomer ratio dependent protein binding have been reported for other drugs of the NSAID class. Ketoprofen for example displays stereoselective drug and protein concentration dependent plasma protein binding (Dubois *et al.*, 1993). The percentage of each enantiomer unbound was also higher with racemate than with each enantiomer alone, similar to the trend observed with etodolac. Multiple binding sites for both enantiomers of ketoprofen have also been noted (Rendic *et al.*, 1980).

In our study we increased the amounts of R-ET and used a fixed (50 μg/mL) concentration of S-ET to determine the effect on unbound fraction of S-ET. There was a trend to increased free fraction of S-ET with increasing concentrations of R-ET (Figure 18). The most probable cause for this observation is displacement of the S-ET from its site I binding on albumin by R-ET. As concentrations of R increase, the free fraction of S increase due to displacement from site I, the site shared by both S and R-ET on human serum albumin.

Tissue Distribution

Tissue distribution 4 h following dosing with a fixed amount of S-ET (5 mg/Kg) and 1,3, and 5 mg/kg of R-ET were determined for plasma, heart, kidney, liver duodenum, and stomach. (Figure 19, Figure 20, Figure 21, Figure 22, Figure 23,

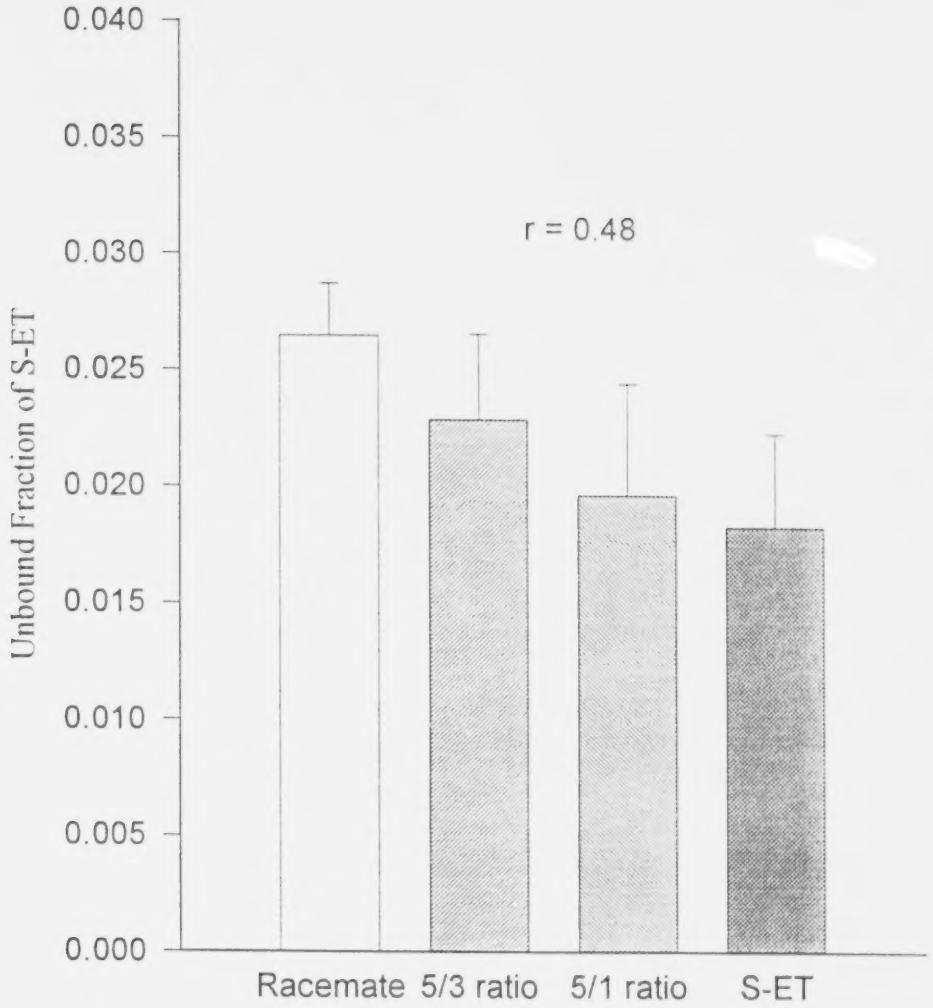

Figure 18. Plasma protein binding in pooled human plasma for a fixed concentration of S-ET (50μg/mL) and increasing concentrations of R-ET enantiomers.

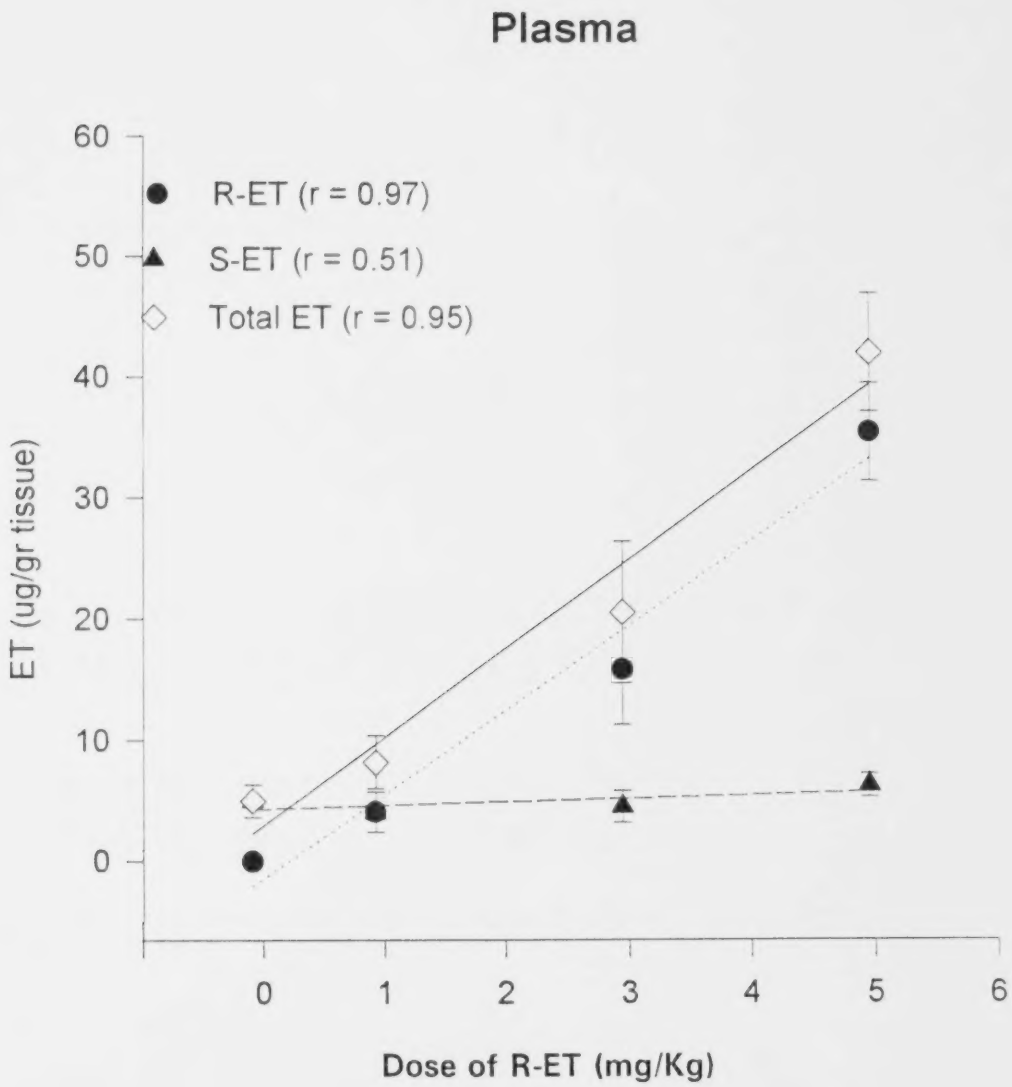

Figure 19. Tissue distribution into plasma 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point).

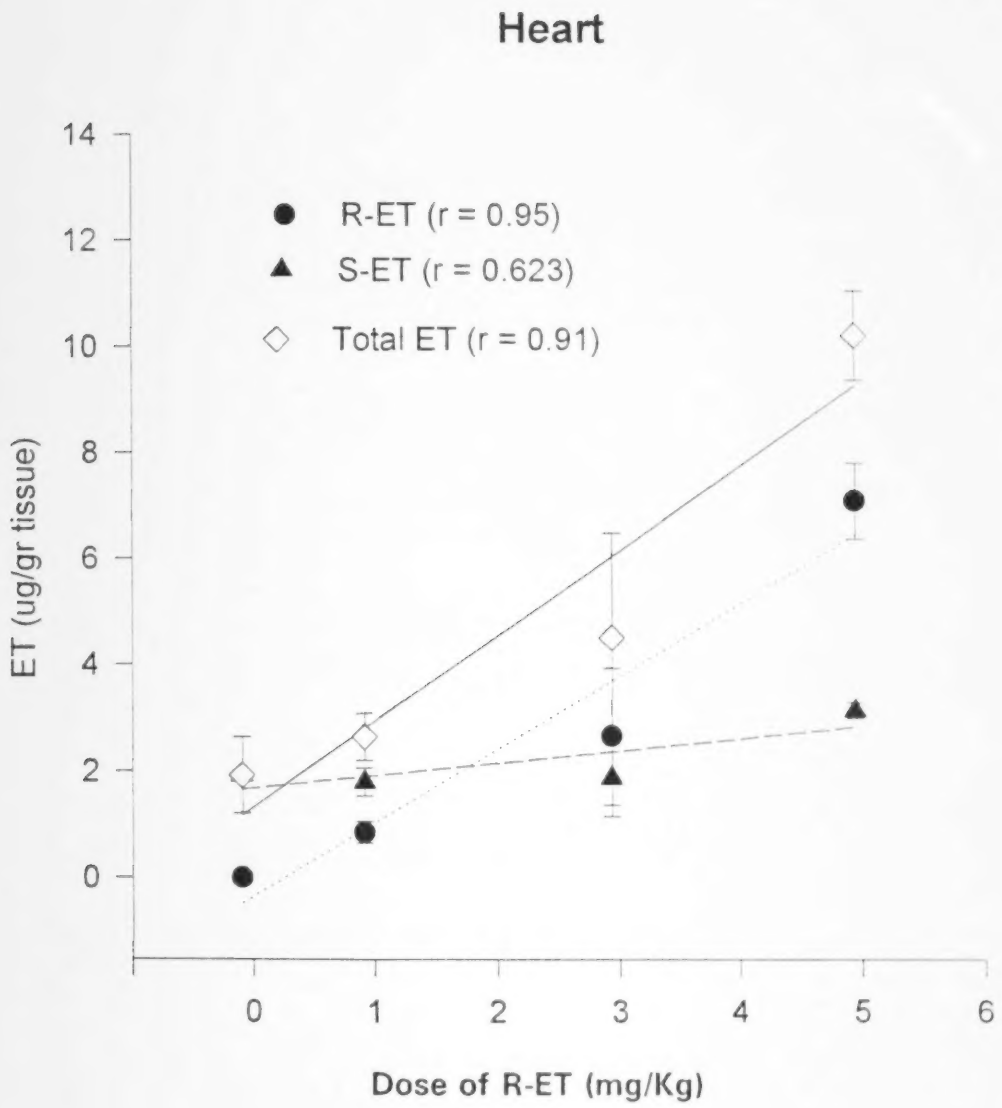

Figure 20. Tissue distribution into heart 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point)

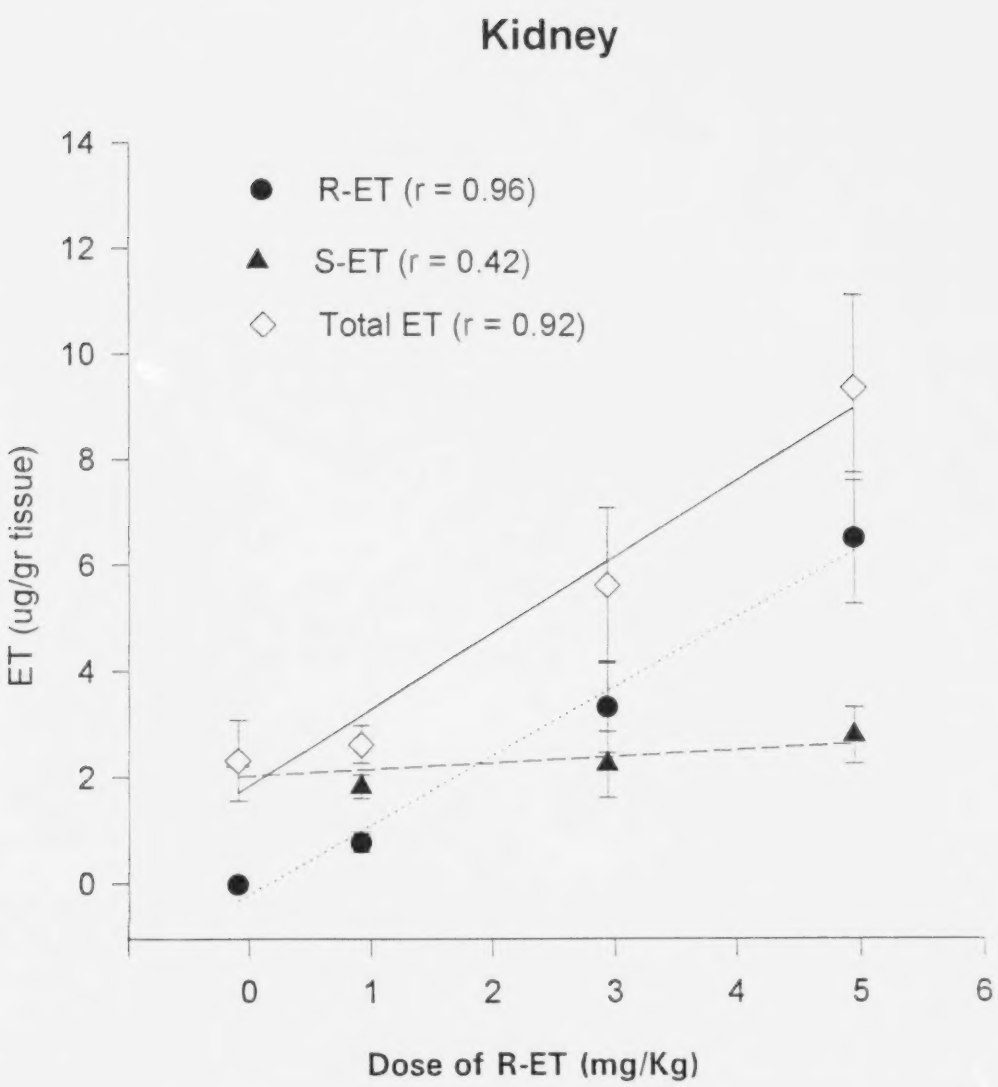

Figure 21. Tissue distribution into kidney 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point)

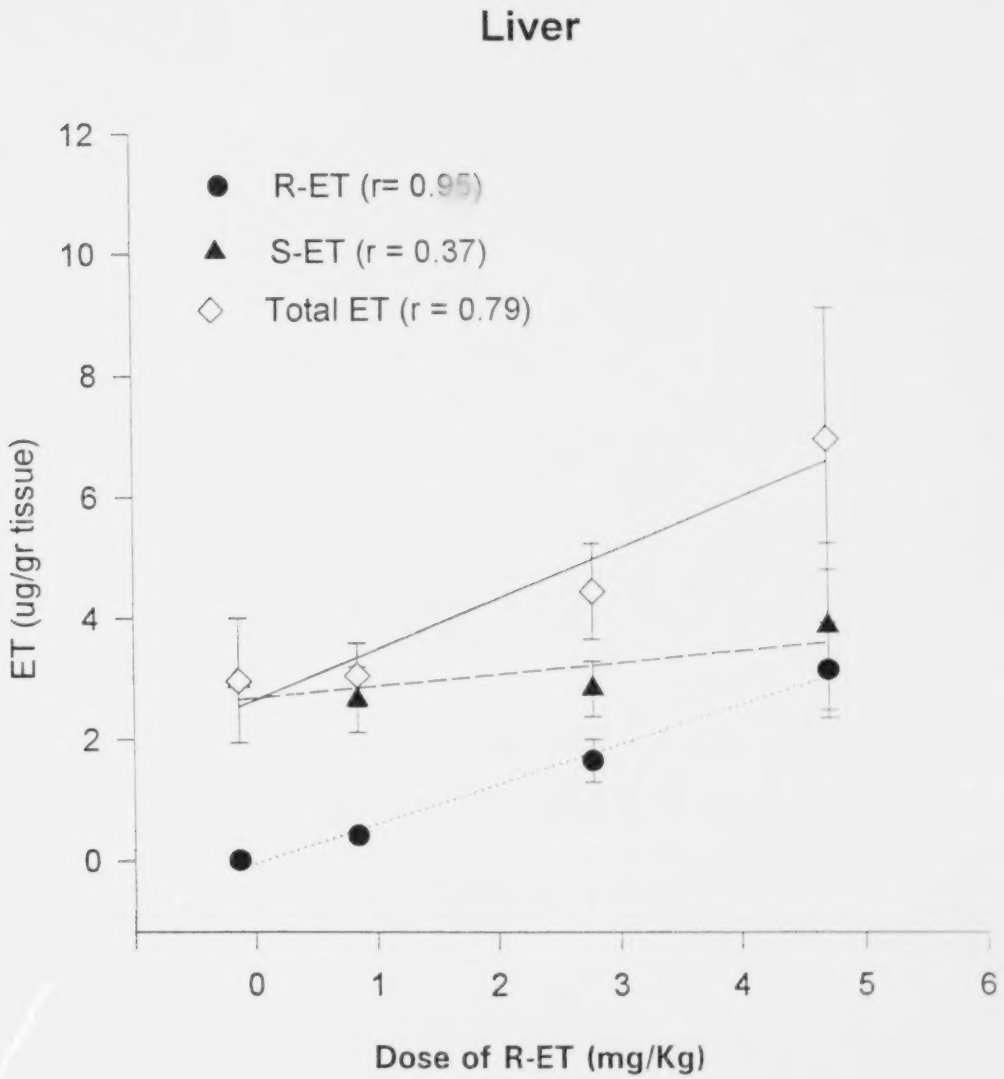

Figure 22. Tissue distribution into liver 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point)

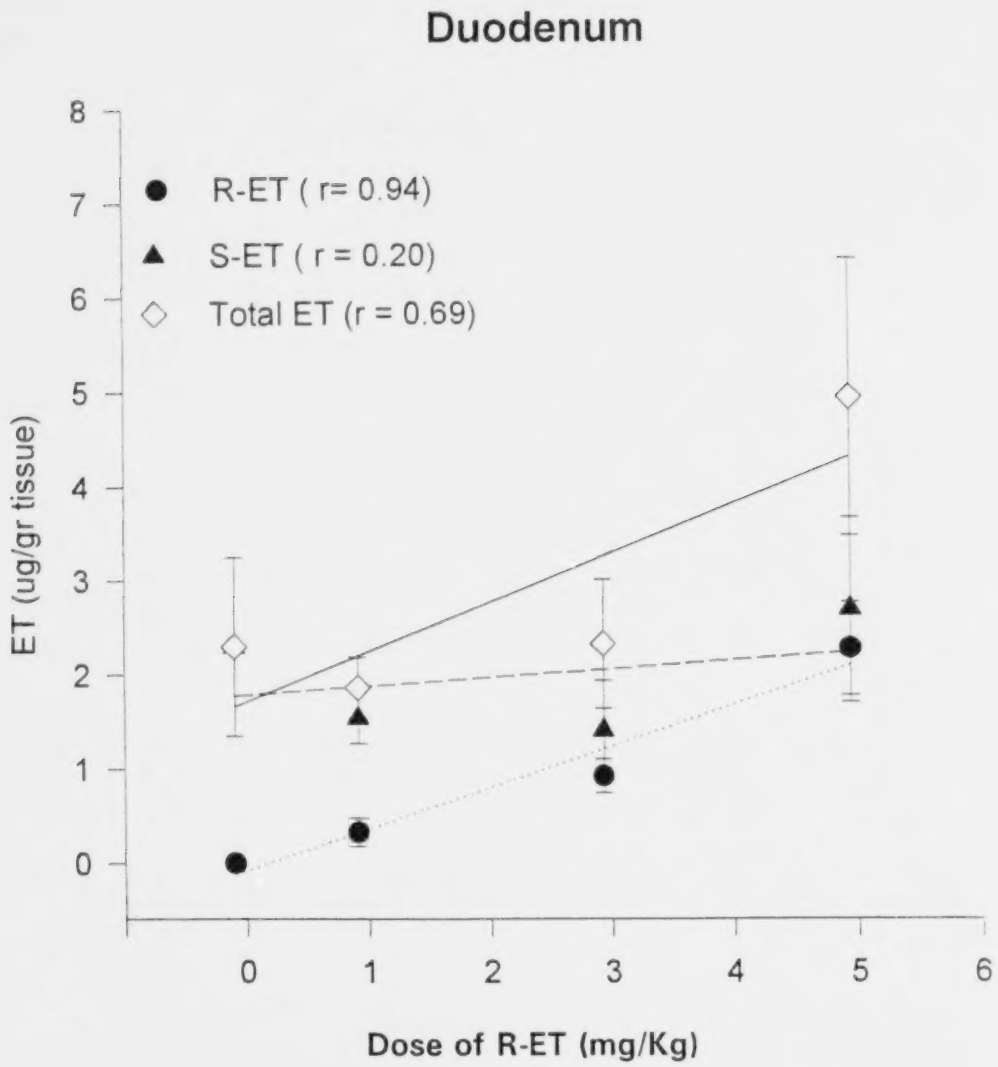

Figure 23. Tissue distribution into duodenum 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point)

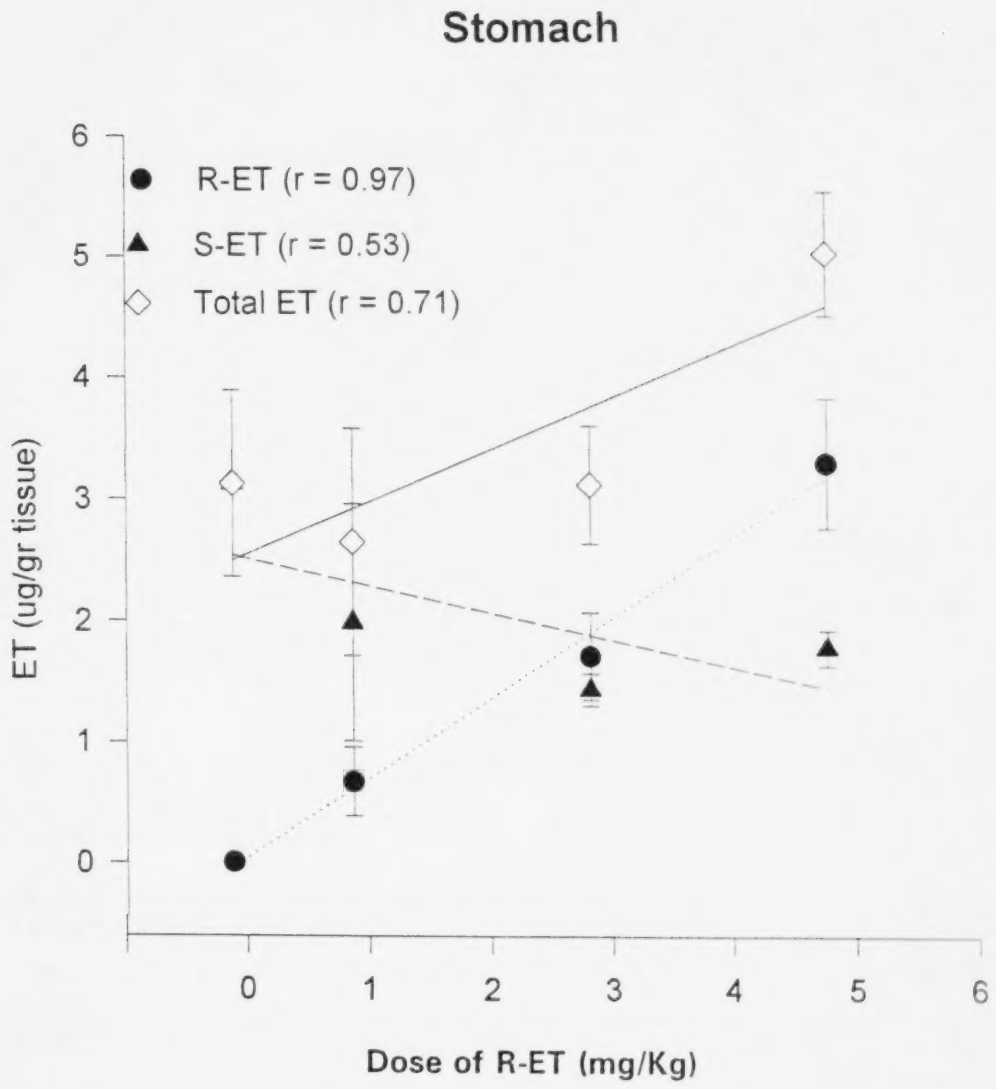

Figure 24. Tissue distribution into stomach 4 h post-dosing with 5 mg/kg of S-ET and 1,3,5 mg/kg of R-ET (n = 4 at each point)

Figure 24). Tissue distribution studies on jejunum and ileum were also attempted but were unavailable due to interference by endogenous compounds in these tissues.

The concentrations of ET observed in tissue were similar to those observed earlier using the same dose of S-ET. (Brocks and Jamali, 1991). Concentrations of R-ET correlated well with the dose of R-ET given. The correlation of total dose also correlated well with the total dose given, but, as expected, to a lesser degree than for R-ET.

Table 7 Relations (r) between amount of ET in tissue (μg/gr) and dose given

	Plasma	*Heart*	*Kidney*	*Liver*	*Duodenum*	*Stomach*
R-ET Dose	0.97	0.95	0.96	0.95	0.94	0.97
Total-ET Dose	0.95	0.91	0.92	0.79	0.69	0.71

Table 8 Relations (r) between amount of S-ET in tissue (μg/gr) and total ET dose (5-10 mg/kg) given (p value in brackets are from regression analysis)

	Plasma	*Heart*	*Kidney*	*Liver*	*Duodenum*	*Stomach*
Total dose	0.51	0.62	0.42	0.37	0.2	0.26
	(0.04)	(0.01)	(0.10)	(0.21)	(0.44)	(0.04)

Heart and stomach showed significant differences between various compounds in the amount of the active S-ET present in each tissue (ANOVA, $p = 0.04$ and 0.01 rspectively). In heart tissue, the concentration of S-ET was significantly higher after

the racemate as compared with all other ratios. In stomach, concentrations were lowest with the racemate. Kidney and duodenum showed similar trends to that of heart, with concentrations of the racemate and S being the highest.

It has been postulated that the amount of free drug in plasma is the controlling factor in the distribution of ET to tissues (Brocks and Jamali, 1991). An attempt was made to correlate tissue concentration of S-ET with total increasing dose of ET (Table 8). Although the variance in the S-ET tissue data was only explained to a small degree by a linear correlation with increasing dose, it was significant for plasma, heart and stomach tissues. This suggests that for at least these 3 tissues in the rat, the presence or absence of R-ET plays a role in distribution of S-ET into tissue, albeit to a minor extent. Other factors important in tissue distribution may also play a role in explaining the observed differences in tissue distribution and the tissue dependent manner in which it occurred. Such factors as organ pH, fat, protein and extracellular water content, and degree of organ perfusion, may have been responsible for the large number of patterns of tissue distribution present.

3.3.3 Intestinal toxicity

Lower intestinal toxicity time course

Both S-ET and racemic ET resulted in increased G.I. permeability to ^{51}Cr-EDTA (Figure 25). R-ET did not increase permeability significantly above baseline at any time points. S-ET appeared higher than racemate throughout the permeability time

profile, and significantly so at 6 and 12 h. All plots showed secondary maxima similar to plasma concentration vs time profiles of ET.

The lower intestinal safety of ET in the rat model has been compared to other NSAIDs by a number of authors (Melarange *et al.*, 1994, Davies,1996). It appears that ET is as safe as a number of other compounds (Davies, 1996). However, it has been reported that ET may be less safe than newer NSAIDs, such as nambumetone (Melarange *et al.*, 1994). These differences were suggested to be due to differences in acidic nature and differential effects on prostanoid production. ET maintains a 10 fold selectivity for COX II versus COX I isoenzymes, lower than that of nabumetone (Glaser *et al.*, 1995). Nevertheless, various reports indicate that ET demonstrates superior safety in humans over a number of other marketed NSAIDs (Table 3).

Stereoselective intestinal toxicity of ET has also been examined (Wright *et al.*, 1994) and it has been observed that S-ET alone was as toxic or more than the same dose of S-ET given as the racemate. Similar results were observed after both a single dose (Figure 29) and as determined over a time course of toxicity time period (Figure 28), although these results did not reach significance for the single dose study. One difference however, was that Wright *et al.* observed that R-ET induced significant permeability changes, which we did not observe (Figure 25).

Pharmacokinetic-pharmacodynamic links

It was noted that intestinal toxicity lagged behind plasma concentrations from 2 to 4 h (Figure 26). The resultant concentration effect relationship, therefore, displayed

counter-clockwise hysteresis typical of effects that have a lag period (Figure 27).

Theoretically, a temporal displacement in effect can be a result of an active metabolite, delay in equilibrium of drug to the site of action, or lag in the time for effect (Holford and Sheiner, 1981). Since ET is known to have no important active metabolites, and the secondary maxima for enterohepatic circulation is observed 2- 4 h before the secondary maxima in the effect curve, the most likely causes are delay in the toxicological effect, and/or delay in equilibrium (Figure 26).

The effect vs concentration-time curves of drugs that exhibit hysteresis in the concentration effect model in the absence of steady state conditions have been studied through the application of models that link pharmacokinetics and pharmacodynamics using a hypothetical effect compartment (Holford and Sheiner, 1981). These compartmental models predict drug concentration in a secondary compartment, and function to shift the time domain of plasma drug concentrations to the right. This allows for effect compartment concentrations and effect to correlate better than plasma concentrations and effect, and useful pharmacodynamic estimates (such as E_{max} and EC_{50} for a sigmoid E_{max} model) may be obtained.

The kinetic-dynamic relationship of NSAIDs are atypical in that the site of toxicity (effect) is also the absorption site for the drug. Furthermore, for ET, the on-going absorption from enterohepatic circulation acts to input drug directly into the effect compartment throughout the effect time course. Thus a new model with direct zero order input into the effect compartment has been developed to simulate the input into the gut from enterohepatic circulation (Vakilynejad 1996) (Figure 8). The

predicted effect compartment concentrations and permeability changes were then fit to the most appropriate pharmacodynamic models.

The effect compartment model yielded a rate constant for equilibrium between compartments (K_{e0},) of 0.334 h^{-1}. The equilibrium half time, calculated from $\ln(2)/K_{e0}$ was 2.1 h, similar to the lag time between effect and concentration (Figure 26).

The parameters of E_{max}, EC_{50}, and γ for the sigmoid E_{max} model were determined for S-ET after dosing with racemate (20 mg/Kg) and with S-ET (10 mg/Kg) (Table 9). Although the E_{max} for S-ET alone was higher than that for S after racemate, in both cases, fits to the E_{max} model were poor, as is clear from the error terms for each parameter. This suggests that the E_{max} model may not be appropriate in this case.

In the case of the linear effect model, a better collapse in the hysteresis was possible (Figure 28). Correlations between concentration of S-ET and lower intestinal permeability to ^{51}Cr-EDTA for ET administered as the racemate (r = 0.66) or S-ET administered alone (r = 0.85) were somewhat better than those observed in plasma (r = 0.38 and 0.29 for racemate and S respectively). The slope for S was greater than that for racemate (Table 10), as indicated by the confidence interval for the slope parameters. This was true even when a zero point was included in the slope calculation for the racemate. Again, this model suggests that S-ET may be more toxic than racemic ET.

Table 9. Estimates of parameters for E_{max} models of effect compartment concentrations vs effect

Dose	*Parameter*	*Estimate*	*S.E.*
Racemate	E_{max} (% ^{51}Cr- EDTA urinary excretion)	8.56	197.
20mg/Kg	EC_{50} (ng/mL)	488.73	731
	Gamma	0.28	2.62
S-ET	E_{max} (% ^{51}Cr- EDTA urinary excretion)	11.98	35.3
10mg/Kg	EC_{50} (ng/mL)	75.27	914
	Gamma	0.49	0.78

These findings agree with those of other authors who also observed larger increases in permeability after dosing with S -*Et al.*one than with the racemate (Wright *et al.*, 1994)

The less than ideal correlation in effect compartment vs. effect linear modeling is probably a result of the fact that the model was incapable of allowing for enterohepatic circulation in the kinetic compartment, thus inaccurately reflecting concentrations. In this model, concentrations were derived from estimated pharmacokinetic parameters. We were unable to fit a multiple enterohepatic recirculation model to the data. This factor greatly increased the inaccuracy of the effect compartment concentrations. The model did however predict effect better than plasma concentrations alone.

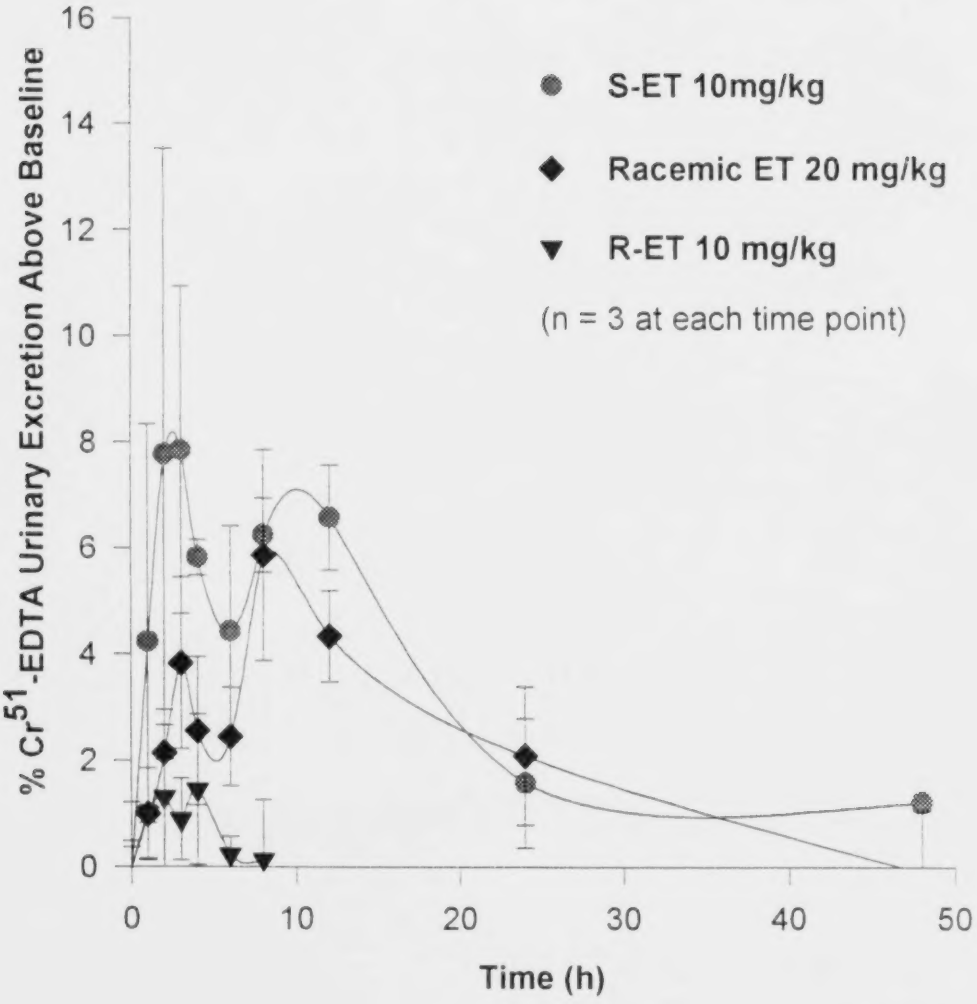

Figure 25. ^{51}Cr-EDTA permeability vs. time profile of S, R and Rac-ET following dosing with 10 mg/kg of S and R, and 20 mg/kg of racemate (n = 4 at each point)

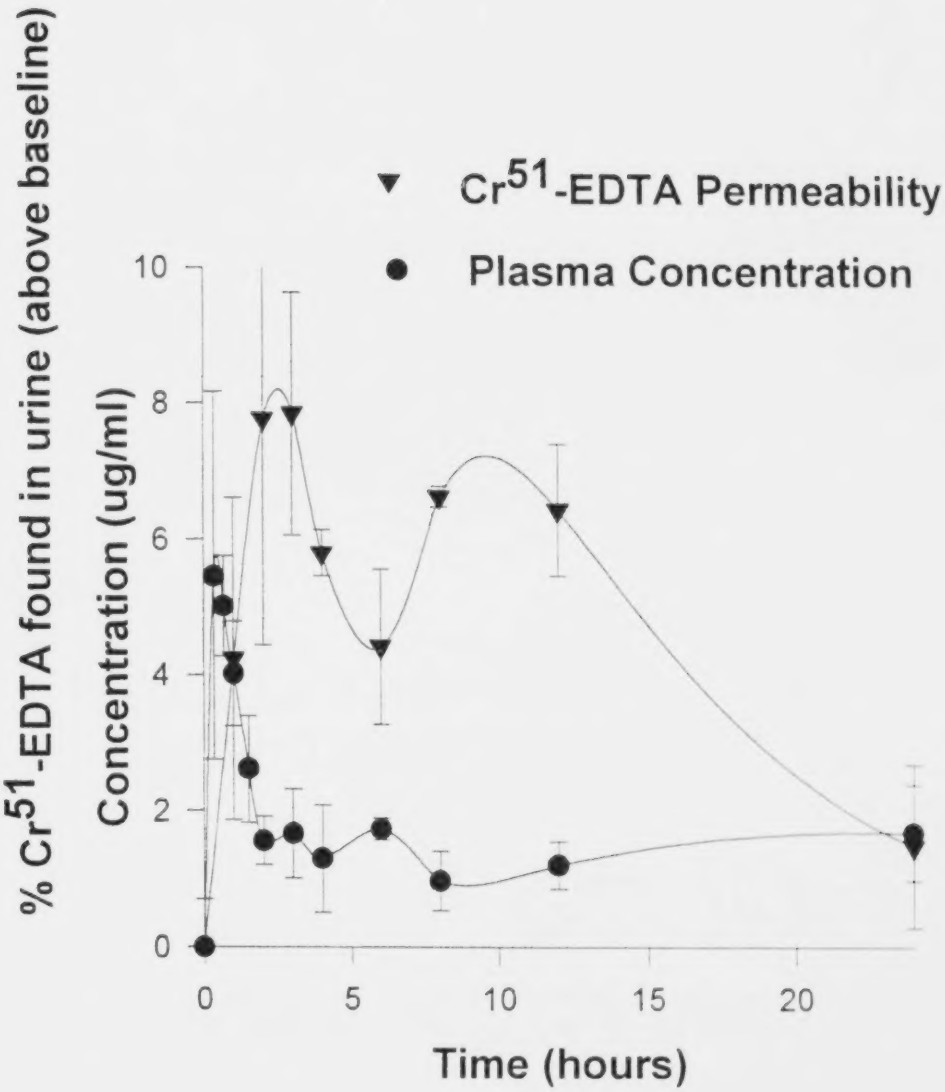

Figure 26. ^{51}Cr-EDTA permeability of S-ET (10mg/kg) and plasma concentration (10mg/kg) vs time (n =4 at each point).

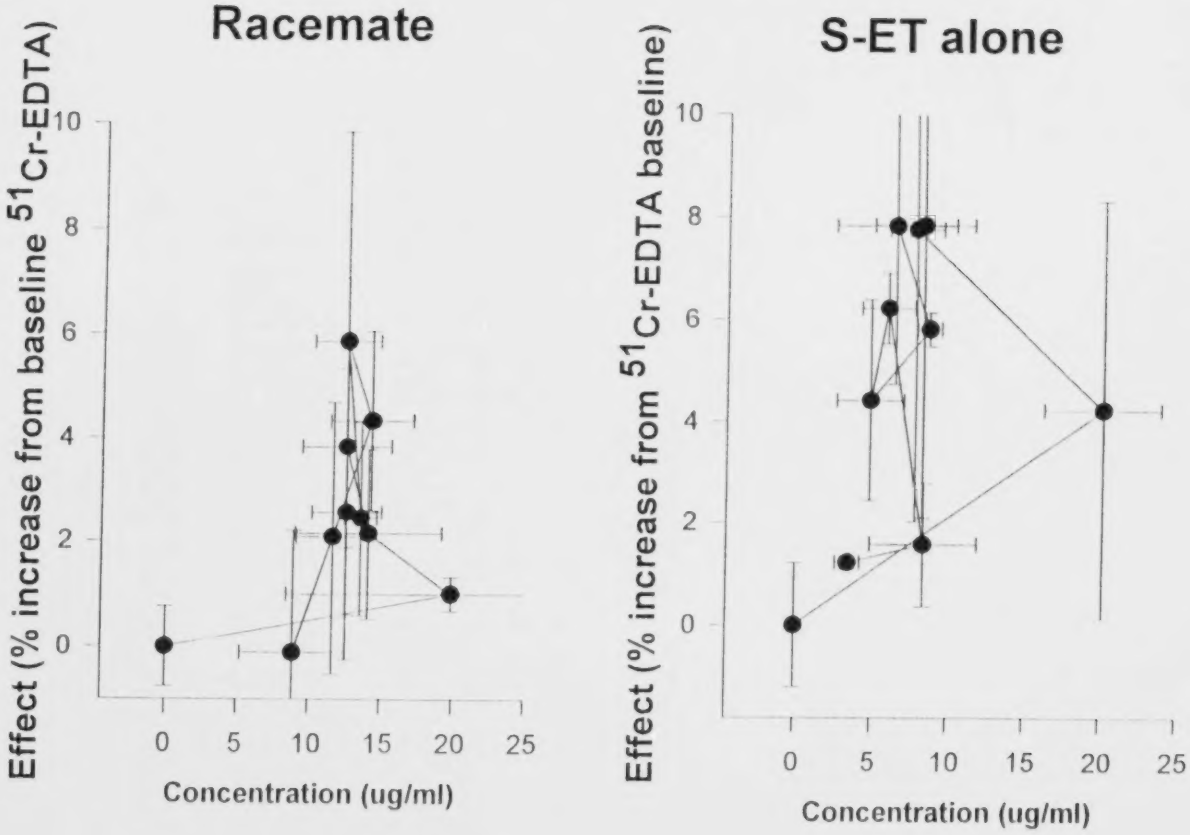

Figure 27. Concentration effect (^{51}Cr-EDTA permeability) relationships for racemate 20mg/kg and S-ET (10mg/kg) (each point is mean and standard deviation of n= 4 observations of both concentration and effect)

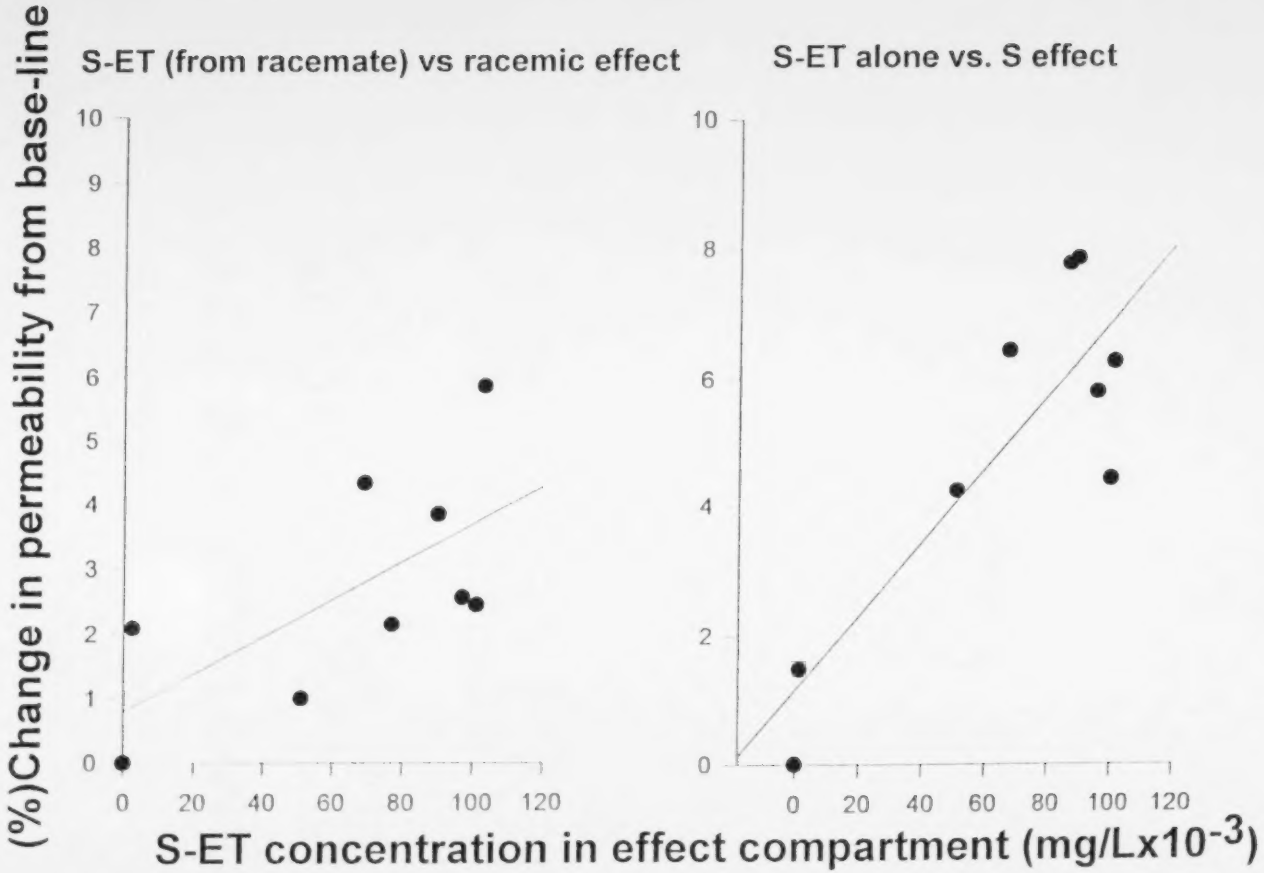

Figure 28. Concentration in effect compartment vs effect (^{51}Cr-EDTA permeability) relationships for racemate 20mg kg and S-ET (10mg kg) (N = 9).

Table 10 Estimates of parameters for linear effect model of effect compartment concentrations vs effect

Dose	*Parameter*	*Estimate*	*S.E.*	*Confidence Interval (95 %)*
Racemate 20 mg/Kg	Slope	0.03	0.01	0.00 to 0.06
	Intercept	0.79	0.95	-1.44 to 3.03
S-ET 20 mg/Kg	Slope	0.06	0.01	0.03 to 0.09
	Intercept	1.00	1.01	- 1.38 to 3.38

Intestinal toxicity with various ratios

Following doses with various enantiomeric ratios of ET, all treatments increased permeability significantly above baseline. Significant inter-group differences were observed in the intestinal permeability to ^{51}Cr-EDTA ($p=0.025$). The percent increase in permeability from baseline, in rank order (mean ± S.D.) was 5/3 (260±170%), 5/1 (197±148%), 5/0 (119±152%), and 5/5 (98±123%). The exact mechanism behind these changes remains unclear. Nevertheless, the changes observed can not be explained by an additive toxicologic effect of the enantiomers since ratios that contained more total drug yielded lower permeability changes than did those that contained less. As well a mechanism based on a competitive process between the active S-ET and the inactive R-ET would yield a graph with toxicity of pure S-ET being the highest which was not

the case. In this study the toxicity of S-ET was also higher than that of the racemate, similar to observation of others (Wright *et al.*, 1994) although it did not reach significance.

The above results cannot be explained by observations made from physicochemical properties, or tissue distribution. The changes in physicochemical properties in solubility from altered enantiomeric ratio are limited to saturated conditions, as conventional thermodynamics suggests. As well, no differences in partitioning for ET compounds were noted at physiological pH. The patterns of tissue distribution were varied for each tissue, but did not match the pattern of toxicity observed here. A plausible (yet unproven) hypotheses is that a combination of factors, such as relative affinity of the individual tissues for each of the enantiomers, changes in blood perfusion patterns (via changes in the effect of the drug on prostaglandin controlled hemodynamics), and enantiomeric interaction at the receptor site(s) responsible for toxicity may all be responsible for the observed differences in a tissue dependent manner. Another plausible mechanism is a mixed partial agonist/agonist model of effect.

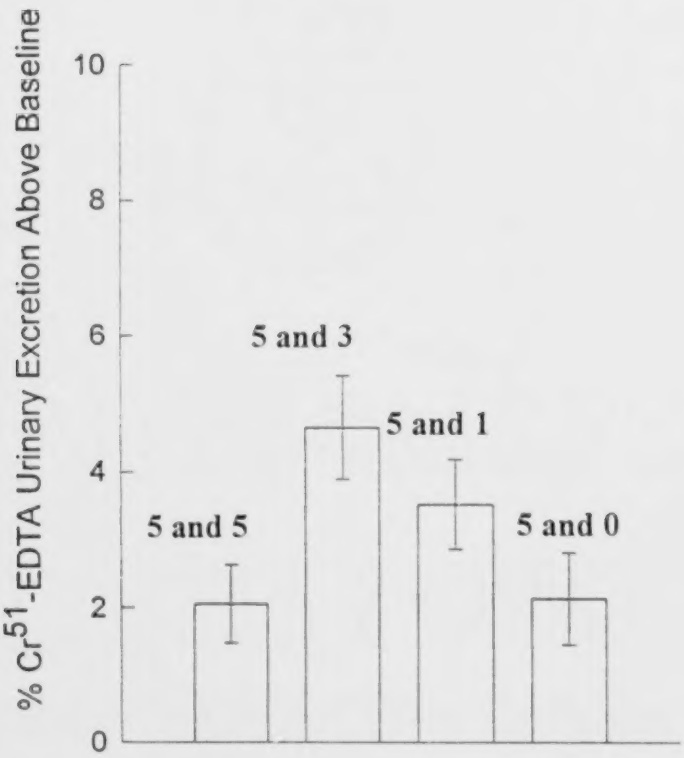

Figure 29. Permeability to ^{51}Cr-EDTA after administration of 5 mg/kg of S-ET and 0, 1, 3, and 5 mg/kg of R-ET. (n = 16 at each point)

4. Conclusions

Minimal sex differences in the pharmacokinetics of ET in Sprague Dawley rats were noted. The primary differences observed were in the excretion of conjugated metabolites of ET (the primary metabolite in rats) into urine, with females having larger amounts of both than males. These difference could be reversed by gonadectomy and sex steroids, specifically,

testosterone. The results suggest that the sex differences in conjugates are not due to prenatally "imprinted' differences in the metabolism of ET, but rather in the maintenance of existing metabolic capacities by androgenic factors in the hypothalmic-pituitary axis.

There was no apparent sex related differences in the lower intestinal toxicity of ET as measured by changes in permeability to ^{51}Cr-EDTA. The effects were also not sex-steroid or gonad dependent, suggesting that the mechanisms by which ET manifests lower intestinal toxicity is not sex dependent. The underlying sex differences in the pharmacokinetics of ET were not sufficient to cause any differences in the observed toxicity of ET. This lack of observed sex differences may not have been apparent due to the dose of ET used and the relativly safe nature of ET.

Due to the minimal differences in kinetics, and lack of differences in lower intestinal toxicity, we concluded that either sex is suitable for use as an animal model for ET studies. We chose to continue our studies with males however, so that the large body of pre-existing knowledge about the pharmacokinetics in male rats could be used as a reference point for further studies involving alterations of enantiomeric ratio.

Altering enantiomeric ratio of Et altered the physicochemical properties of resulting compounds. ET demonstrated the properties of a crystalline addition compound, with 3 distinct crystal forms as determined by DSC. The S, R and racemic crystals formed by various ratios of ET resulted in a binary phase melting point diagram with 2 distinct eutectic points. Melting point could be linearly related to molar fraction by use of thermodynamic equations. Ratio specific solute-solvent interactions in aqueous solutions were observed. No ratio specific alterations in partitioning were noted, except at those pH where solute-solute interaction, such as dimerization was likely to occur.

Although the relationships between enantiomers and their physicochemical properties are well established in general chemistry, there has been virtually no attempt to capitalize on this from a pharmaceutical perspective, as evidenced by the paucity of literature pertaining to this subject. For example, the potential for enhanced drug delivery of chiral compounds from extravascular administration routes, especially transdermal, is considerable.

The effect of altering enantiomeric ratio on the plasma time course of ET was not as pronounced as the effects on physicochemcial properties. The dose vs. AUC relationship for R-ET was linear when increasing doses of R-ET were given with a fixed dose of S, suggesting no changes in clearance of R-ET. As well, no differences in the clearance of a fixed dose of S-ET with increasing doses of R-ET were detected. A trend to increasing protein unbound fraction of S was noted with increasing concentrations of R. This is most likely due to the fact that both S and R share binding site I on serum albumin, the primary protein for ET binding in plasma. Increased concentrations of R may

result in displacement of S-ET. However over the concentration range used in this study, these alterations were minimal.

When tissue distribution was examined, amounts of R-ET in tissue increased linearly with increasing doses of R-ET. S-ET was also altered slightly in a tissue specific manner with increasing doses of R-ET in some tissues. These differences in S-ET are most likely due to differences in tissue affinity for each of the enantiomers, as well perhaps by small changes in protein binding caused by displacement of S-ET by increasing amounts of its antipode.

The permeability time courses of ^{51}Cr-EDTA following dosing with R, S, and racemic ET were studied to determine if enantiomeric interaction occurs in the lower intestinal toxicity of ET. Permeability after dosing with R did not increase significantly above baseline at any time point. However, both racemate and S significantly increased permeability at various time points. These alterations returned to baseline within 48 hours of dosing. S proved to be as toxic or more than racemate. Both showed secondary maxima in their permeability time profiles, similar to those seen in concentration time profiles, but lagging by 2-3 hours. The resultant effect concentration relationships showed hysteresis. This hysteresis was minimized using a pharmacokinetic/pharmacodynamic model with direct zero order input into the effect compartment to mimic enterohepatic circulation, allowing for meaningful correlations between concentrations of ET and effect to be made.

The permeability of ^{51}Cr-EDTA following dosing with various ratios of ET showed significant inter-group differences with a 5/3 S/R ratio having the highest toxicity. These results are not explained by either the pharmacokinetic or physicochemical

alterations that occur by varying enantiomeric ratio of ET. The exact mechanism for these differences is unknown.

Much of our appoach to chiral drug development is based on past practices. Historically, most chiral drugs were derived from natural sources, and as a result, were often optically pure. With increased synthesis there has been an increase in the number of racemic products marketed. Interaction studies for racemics were completed by comparing the racemate to single enantiomer formulations. There is again increased interest in developing pure enantiomer formulations as the technology for separation, analysis, and synthesis of optically pure enantiomers increases.

Using ET as a model we have raised the question: "Why only consider optically pure enantiomer or a 50/50 mixture of each enantiomer for pharmaceutical use ?" We have drawn attention to the fact that physicochemical changes occur when enantiomeric ratio is altered, and that physicochemical properties important to drug formulation and disposition can be optimized. We have demonstrated that for various properties, (such as melting point and lower intestinal toxicity) the racemate and enantiomer may seem similar in properties, but differences may occur at ratios between these two. We have demonstrated that an alternative to racemate/pure enantiomer interaction studies are those that utilize multiple ratios so that interaction can be assessed at more than two points. This allows for a complete assessment of enantiomeric interaction, and for rational and scientific selection of an optimal enantiomeric ratio, based on physicochemical, pharmacokinetic, and pharmacodynamic considerations.

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